EUROPEAN PATENT APPLICATION

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(54) cDNA clones coding for polypeptides exhibiting multi-lineage cellular growth factor activity and/or mast cell growth factor activity.

(57) Plasmid vectors are provided that carry complementary DNA (cDNA) clones coding for polypeptides exhibiting mammalian multi-lineage cellular growth activity and/or mammalian mast cell growth factor activity. One of these polypeptides is 166 amino acids in length, including a potential leader sequence of about 19 amino acids. The cDNA is derived from messenger RNA isolated from a mouse T-cell line after activation with concanavalin A. The cDNA was cloned by incorporation into a plasmid vector, which then transformed into E. coli. The plasmid vector also contained DNA segments from the SV40 virus, permitting expression of the cDNA after transfection into a mammalian host cell, such as monkey COS-7 cells.

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cDNA CLONES CODING FOR POLYPEPTIDES
EXHIBITING MULTI-LINEAGE CELLULAR GROWTH FACTOR
ACTIVITY AND/OR MAST CELL GROWTH FACTOR ACTIVITY

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This invention relates generally to the application of recombinant DNA technology to elucidate the control mechanisms of the mammalian immune response and, more particularly, to the isolation of deoxyribonucleic acid (DNA) clones coding for polypeptides exhibiting multi-lineage cellular growth factor activity and/or mast cell growth factor activity.

Recombinant DNA technology refers generally to the technique of integrating genetic information from a donor source into vectors for subsequent processing, such as through introduction into a host, whereby the transferred genetic information is copied and/or expressed in the new environment. Commonly, the genetic information exists in the form of complementary DNA (cDNA) derived from messenger RNA (mRNA) coding for a desired protein product. The carrier is frequently a plasmid having the capacity to incorporate cDNA for later replication in a host and, in some cases, actually to control expression of the cDNA and thereby direct synthesis of the encoded product in th host.

This technology has progressed xtrem ly rapidly in recent years, and a variety of exogenous proteins have been xpressed in a vari ty of hosts. By

way of example, some of the eukaryotic proteins so-
produced include:
proinsulin (Naber, S. et al., Gene 21: 95-104 [1983]);
interferons (Simon, L. et al., Proc. Nat. Acad. Sci.
U.S.A., 80: 2059-2062 [1983] and Derynck, R. et al.,
Nucl. Acids Res. 1: 1819-1837 [1983]); and growth
hormone (Goeddel, D., et al., Nature 281: 544-548
[1979]). (These publications and other referenced
materials have been included to provide additional
details on the background of the pertinent art and, in
particular instances, the practice of invention, and are
all incorporated herein by reference.)

For some time, it has been dogma that the
mammalian immune response was due primarily to a series
of complex cellular interactions, called the "immune
network". While it remains clear that much of the
response does in fact revolve around the network-like
interactions of lymphocytes, macrophages, granulocytes
and other cells, immunologists now generally hold the
opinion that soluble proteins (e.g. the so-called
lymphokines) play a critical role in controlling these
cellular interactions.

Lymphokines apparently mediate cellular
activities in a variety of ways. They have been shown
to have the ability to support the proliferation and
growth of various lymphocytes and, indeed, are thought
to play a crucial role in the basic differentiation of
pluripotential hematopoietic stem cells into the vast
number of progenitors of the diverse cellular lineages
responsible for the immune response. Cell lineages
important in this response include two classes of
lymphocytes: B cells, which can produce and secrete
immunoglobulins (proteins with the capability of
r cognizing and binding to foreign matter to ffect its

removal), and T cells (of various subsets) that induce
or suppress B cells and some of the other cells
(including other T cells) making up the immune network.

Another important cell lineage is the mast
cell - a granule-containing connective tissue cell
located proximate to capillaries throughout the body,
with especially high concentrations in the lungs, skin,
and gastrointestinal and genitourinary tracts. Mast
cells play a central role in allergy-related disorders,
particularly anaphylaxis, and this role can be briefly
stated as follows: once certain antigens crosslink
special immunoglobulins bound to receptors on the mast
cell surface, the mast cell degranulates and releases
the mediators (e.g., histamine, serotonin, heparin,
kinins, etc.) which cause allergic reactions, e.g.
anaphylaxis.

Research to better understand (and thus
potentially treat therapeutically) allergy, anaphylaxis
and other immune disorders, through the study of mast
cells, T cells and the other cells involved in the
immune response, has been hampered by the general
inability to maintain these cells _in vitro_. However,
several immunologists recently discovered that such
cells could be isolated and cultured by growing them on
secretions from other cells, e.g., conditioned media
from splenic lymphocytes stimulated with Concanavalin A
(ConA). It has now become clear from this work that the
generation of cell clones is dependent on specific
factors, such as lymphokines.

Apparently almost all blood cell types are
continuously generated in the adult vertebrate bone
marrow through the growth and differentiation of the
hierarchy of hematopoietic progenitor cells. At the
apex of this hierarchy is the pluripotent stem cell,

which can repopulate a lethally irradiated animal for
most, if not all, immunological cell types (e.g., red
cells, platelets, lymphocytes, various granulocytes and
monocytes/macrophages). The pluripotent cell not only
has the capacity to regenerate the pluripotent stem cell
compartments (self-renewal), but also gives rise to
progenitor cells committed to development along one
particular lineage pathway. Progeny of a particular
committed stem cell appear to share the same lineage
commitment as the parent cell (Metcalf, D., "Hemopoietic
Colonies", Springer Publishing Co., New York, N.Y.
[1977]).

In vitro studies on hematopoiesis have shown
that a number of soluble colony stimulating factors
(CSF) can regulate the growth of these various
progenitor cells. Some of these factors have been
partially purified and shown to affect specifically stem
cells belonging to a particular cell lineage. For
example, erythropoietin stimulates more-differentiated
members of the erythroid hierarchy (Miyake, T., et al.,
J. Biol. Chem. 252: 5558 [1977]), and another factor
(colony stimulating factor-macrophage or CSF-1)
preferentially stimulates macrophage growth in semi-
solid cultures of bone marrow cells (Stanley, E., and
Heard, P., J. Biol. Chem. 252: 4305 [1977]). Yet
another type of growth factor seems able to stimulate
hematopoietic colonies consisting of single cell types
and mixtures of cells. The range of cells, e.g.
erythrocytes, megakaryocytes, granulocytes, mast cells
and monocyte/macrophages, that are responsive to one
factor of this second typ have caused it to be named a
multi-lineage cellular growth factor (multi-CSF)
(Iscove, N. et al., J. Cell. Physiol. Suppl., 1: 65-78
[1982]), indicating its capability of affecting a number

of committed progenitor cells, and perhaps
pluripotential stem cells as well.

One of the better characterized factors is
interleukin-1 (IL-1), a factor released from
macrophages, which induces replication of thymocytes and
peripheral T cells (Mizel, S. et al., J. Immunol. 120:
1497-1503 [1978]). Similarly, interleukin-2 (IL-2) and
interleukin-3 (IL-3) are two well-studied lymphokines
released by certain stimulated lymphocytes. A very
significant characteristic of IL-2 is its ability to
support the continuous growth of certain T cells in
vitro (Farrar et al., Ann. N.Y. Acad. Sci. 332: 303-15
[1979]). Likewise, an important characteristic of IL-3
is its ability to support the growth of cell lines
having the phenotypic characteristics of mast cells
(Ihle, J. et al., Immunological Rev. 63: 5-32 [1982]).
A number of other cellular growth properties have been
ascribed to IL-3 as well (see Ihle, J. et al., J.
Immunol. 131: 282-287 and 129: 2431 [1981]), but its
precise relationship with multi-CSF has been unclear.

Whereas both mouse IL-2 and IL-3 have been at
least partially characterized biochemically (Gillis, S.
et al., J. Immunol. 124: 1954-1962 [1980] and Ihle, J.
et al., J. Immunol. 129: 2431-2436 [1982],
respectively), IL-2 is presently the accepted primary
factor responsible for T-cell growth, whereas the
protein(s) responsible for mast cell growth factor
(MCGF) and CSF activity have not been agreed upon to the
same extent. It is now thought that mouse IL-2 has a
molecular weight (probably as a dimer) of approximately
30-35,000 (Simon, P. et al., J. Immunol. 122: 127-132
[1979]), although some variations are recognized (Robb,
R. and Smith, K., Molec. Immun. 18: 1087-1094 [1981]);
and human IL-2 apparently has a molecular weight of

about 15,000 (Gillis, S. et al., Immun. Rev. 63: 167-209 [1982]). Moreover, a cDNA clone coding for human IL-2 has recently been reported (Taniguchi, T. et al., Nature 302: 305-310 [1983]). On the other hand, mouse mast cell growth factors have been variously reported as having molecular weights of 45,000 (Nabel et al., Nature, 291: 332-334 [1981]), of 35,000 (Yung, Y. et al., J. Immunol. 127: 794-799 [1981]) and of 28,000 (Ihle, J. et al., J. Immunol. 129: 1377-1383 [1982]). Similar discrepancies surround the CSF's.

Although such molecular weight differences could perhaps be partially explained by varying amounts of glycosylation, clarification of the issue requires additional structural data, e.g., substantially full-length sequence analysis of the molecules in question. Protein sequencing offers, of course, a possible means to solve the problem, but it is very difficult work experimentally and often can provide neither completely accurate nor full-length amino acid sequences. Moreover, having the capability of making bulk quantities of a polypeptide exhibiting mammalian MCGF or CSF activity will greatly facilitate the study of the biology of mast cells and other cells involved in the immune response; e.g., by minimizing the necessity of relying on ConA-conditioned media for stimulating cell growth. Accurate and complete sequence data on an MCGF or CSF will also serve to simplify the search for other immunological factors. Finally, additional information on any lymphokine will help in evaluating the roles of the various growth factors and cells of the immune network and thus provide insight into the entire immune system - with the concomitant therapeutic benefits.

Thus, there exists a significant need for extensive nucleotide sequence data on the DNAs coding

for, and amino acid sequences of, proteins exhibiting MCGF or CSF activity, as well as a simple and economic method of making substantial and essentially pure quantities of such materials. The present invention fulfills these needs.

The present invention provides cDNA clones coding for polypeptides exhibiting mammalian mast cell growth factor (MCGF) activity and or multi-lineage cellular growth factor activity. A nucleotide sequence for a cDNA and a putative amino acid sequence for an associated polypeptide are shown in Figure 1. The cDNA sequence can be integrated into various vectors, which in turn can direct the synthesis of the corresponding polypeptides in a variety of hosts, including eukaryotic cells, such as mammalian cells in culture.

More specifically, the invention provides a process for producing a polypeptide exhibiting mammalian MCGF activity and/or multi-lineage cellular growth factor activity, the process comprising the steps of:

a) providing a vector comprising a nucleotide sequence coding for said polypeptide, wherein the nucleotide sequence is capable of being expressed by a host containing the vector;

b) incorporating the vector into the host; and

c) maintaining the host containing the vector under conditions suitable for transcription of the nucleotide sequence into said polypeptide.

Preferably, the cDNA sequences are derived from an mRNA sequence coding for the polypeptides, and the host is an organism such as a eukaryotic, e.g. mammalian, cell transfected or transformed with the vector. Further, the vector preferably comprises also a second nucleotide s qu nce capable of controlling

expression of the nucleotide sequence coding for the
polypeptide. This second sequence coding can include a
promoter sequence, one or more intron sequences and a
polyadenylation sequence, to permit, respectively,

5   transcription, splicing and polyadenylation of the
nucleotide sequence coding for the polypeptide.

Particularly, when the host is a mammalian
cell, such as a COS-7 monkey (kidney) cell, the vector
contains the promoter sequence of the simian virus 40

10 (SV40) early region promoter and the polyadenylation
sequence of the SV40 late region polyadenylation
sequence.

The mouse cDNA sequence of Figure 1 (see
below) is capable of hybridizing with other DNA

15 sequences, such as DNA coding for other mammalian growth
factors from a cDNA or genomic library. It is noted
that the described cDNA sequences seem to contain
information for a leader sequence.

The polypeptides of the present invention are

20 capable of enhancing mammalian mast cell and other cell
growth, particularly in in vitro cultures. Suitable
pharmaceutical compositions can be prepared by adding
the polypeptides (preparations of which are essentially
free of other mammalian growth factors) to

25 therapeutically compatible carriers.

Other features and advantages of the invention
will become apparent from the following detailed
description, which describes, in conjunction with the
accompanying drawings and by way of example, the present

30 invention.

In the Drawings:
Figure 1 illustrates the nucleotide sequence
and putative corresponding amino acid sequence of a cDNA

clone exhibiting multi-lineage cellular growth factor activity;

Figure 2 depicts the amount of MCGF activity in fractions of a sucrose gradient sedimentation of mRNA isolated from ConA-stimulated Cl.Ly $1^+2^-/9$ cells. The locations of 18S and 28S ribosomal peaks are indicated;

Figure 3 illustrates pcD-MCGF, a plasmid carrying a cDNA clone exhibiting mast cell growth factor activity and multi-lineage cellular growth factor activity; and

Figure 4 is a restriction endonuclease cleavage map of the cDNA insert of Figure 3.

In Figure 3, transcription of the 950 bp cDNA insert contained in the pcD expression vector from the SV40 early promoter is indicated by the arrow. The locations of the splice donor and acceptor sites are shown. The polyadenylation signal, also derived from SV40, is located at the 3'-end of the cDNA insert. The cDNA insert is heavily shaded. The remainder of the vector sequences are derived from pBR322, including the β-lactamase gene (Amp^R) and the origin of replication.

In accordance with the present invention complementary DNA (cDNA) clones are provided for polypeptides exhibiting mammalian mast cell growth factor (MCGF) activity and/or multi-lineage cellular growth factor (multi-CSF) activity. After the cDNA sequences have been incorporated into replicable expression vectors, and the vectors transfected into an appropriate host (e.g. a mammalian cell culture), the expressed polypeptide or polypeptides will possess the ability to allow the expansion of mast cells and hematopoietic cells to multiple lineages.

An exemplary, putative amino acid sequence
based on the isolated nucleotide sequence is shown in
Figure 1. A portion of the predicted sequence (amino
acids 33 to 41) is identical with the reported NH_2-
5   terminal sequence of mouse Interleukin-3 (IL-3), which
has been shown to exhibit mouse MCGF activity and multi-
CSF activity (Ihle, J. et al., J. Immunol. 129, 2431-
2436 [1982]; Ihle, J. et al., J. Immunol. 131, 282-287
(1983); and Garland, J. et al., Eds. Oppenheim, J. and
10   Cohen, S., "Interleukins, Lymphokines, and Cytosines",
Proceedings of Third Int. Lymphokines Workshop, Academic
Press, New York, pages 123-129 [1983]). The coding
region located between the translation start codon (ATG)
and the beginning of the sequence contained in IL-3 is
15   rich in hydrophobic amino acids, as would be expected
for a leader sequence of a secreted protein. Therefore,
the polypeptide's mature form in vivo, as secreted,
possibly begins with an Asp residue, as does IL-3, and
the preceding 20 or so amino acids - constituting the
20   putative leader sequence - are removed by proteolytic
processing. Assuming this to be accurate, the mature
polypeptide exhibiting MCGF and multi-CSF activities
would consist of 134 amino acids, with a calculated
molecular weight of about 15,000. Furthermore, the
25   presence of four potential N-glycosylation sites, i.e.,
Asn-X-Ser (where X is an amino acid residue) at deduced
amino acid positions 42-44, 70-72, 77-79, and 112-114 of
the polypeptide (see Neuberger et al., Glycoproteins 5,
450-490, Elsevier Publishing Co., U.S.A. [1972]),
30   suggests that it would be glycosylated in vivo.

When transfected into COS-7 monkey cells, one
of the cDNA clones of this invention directs the
synthesis of biologically active MCGF and multi-CSF.
Addition of this expressed cloned gene product to

cultures of mouse bone-marrow cells allows the expansion of hematopoietic cells committed to multiple lineages; it supports the formation of burst-forming erythroid colonies (BFU-E), granulocyte/macrophage colonies (CFU-G/M), mast cell colonies (CFU-mast), as well as colonies of multiple lineages (CFU-Mixed), and sustains multipotential stem cells (CFU-S) in liquid culture.

A variety of methods may be used to prepare the cDNAs of the present invention. By way of example, total mRNA is extracted (e.g., as reported by Berger, S. et al., Biochemistry 18: 5143-5149 [1979]) from a cell line (e.g. a hybrid cell line) producing polypeptides exhibiting mammalian mast cell growth factor activity. The double-stranded cDNAs from this total mRNA can be constructed by using primer-initiated reverse transcription (Verma, I., Biochim. Biophys. Acta, 473: 1-38 [1977]) to make first the complement of each mRNA sequence, and then by priming for second strand synthesis (Land, H. et al., Nucleic Acids Res., 9: 2251-2266 [1981]). Subsequently, the cDNAs can be cloned by joining them to suitable plasmid or bacteriophage vectors (Rougeon, F. et al., Nucleic Acids Res., 2, 2365-2378 [1975] or Scherer, G. et al., Dev. Biol. 86, 438-447 [1981]) through complementary homopolymeric tails (Efstratiadis, A. et al., Cell, 10, 571-585 [1977]) or cohesive ends created with linker segments containing appropriate restriction sites (Seeburg, P. et al., Nature, 270, 486-494 [1977] or Shine, J. et al., Nature, 270, 494-499 [1977]), and then transforming a suitable host. (See generally Efstratiadis, A., and Villa-Kormaroff, L., "Cloning of double stranded cDNA" in Setlow, J. and Hollaender, A. (eds.) Genetic Engineering, Vol. 1, Plenum Publishing Corp., N.Y., U.S.A. [1982].)

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A preferred method of obtaining the full-length cloned cDNAs of this invention is the procedure developed by H. Okayama and P. Berg (Mol. and Cell. Biol., 2: 161-170 [1982]). This method has the advantage of placing the cDNA inserts in a bacterial cloning vector at a position whereby the cDNA can also be directly translated and processed in mammalian cells. Briefly, the first cDNA strand is primed by polydeoxythymidylic acid covalently joined to one end of a linear plasmid vector DNA. The plasmid vector is then cyclized with a linker DNA segment that bridges one end of the plasmid to the 5' end of the cDNA coding sequence. By employing a DNA fragment containing the Simian Virus 40 (SV40) early region promoter and a linker containing a modified SV40 late region intron, the cDNA can be expressed _in vitro_ in COS-7 mouse kidney cells without further modification. (See generally Okayama, H. and Berg, P., Mol. and Cell. Biol., 3: 280-289 [1983] and Jolly, D. _et al._, Proc. Nat. Acad. Sci. U.S.A., 80: 477-481 [1983].)

The desired cDNA clones can also be detected and isolated by hybridization screening with appropriate mRNA samples (Heindell, H. _et al._, Cell, 15: 43-54 [1978]). Alternatively, the cDNA libraries can be screened by hybrid selection (Harpold, M. _et al._, Nucleic Acid Res., 5: 2039-2053 [1978] or Parnes, J. _et al._, Proc. Nat. Acad. Sci. U.S.A., 78: 2253-2257 [1981]) or in _Xenopus_ oocytes (Aurdon, J., Nature, 233: 177-182 [1971]). (See generally Villa-Komaroff, L. _et al._, Proc. Nat. Acad. Sci. U.S.A., 75: 3727-3731 [1978].)

Once the cDNA library in the Okayama/Berg plasmid vector has been completed, the cDNA clones are collected, and random pools are checked for the presence of the desir d cDNAs by hybrid selection, translation,

and assay (e.g. by measuring MCGF or multi-CSF growth factor activity, the existence of antigenic determinants, or other biological activities). Pools positive by these criteria can then be probed with an appropriate subtracted probe, e.g., cDNA from a B cell line and/or uninduced T cell line. Thereafter, the positive, probed pools are divided into individual clones which are tested by transfection into a suitable host (such as a mammalian cell culture), and the host supernatant assayed for the desired activity (e.g. multi-CSF or MCGF activity). Positive clones are then sequenced.

In further describing the procedures relating to preparing cDNA clones of the invention, the mast cell and other lines will be considered first, followed by general descriptions of the procedures of the _in vitro_ translation of mRNA coding for a protein exhibiting MCGF activity; the construction of a cDNA library containing the cDNA sequences; hybrid selection of the library; isolation of full-length cDNA clones in a plasmid vector and subsequent expression in mammalian cells; multi-CSF assays; human multi-CSF and MCGF isolation, subcloning and expression in bacteria and yeast; and purification and formulation. A more detailed description of the entire experimental process will follow thereafter.

Mast Cell and T-Cell Lines

The preferred cells for use in connection with the present invention are those developed as described by Galli, J. _et al._ (J. Cell Biol., 95: 435-444 [1982]). One cloned mast cell line, designated MC/9 and deposited at the American Type Culture Collection (accession number ATCC CRL 8306) was grown in Dulbecco's modified Eagle's medium (DME) supplemented with 5%

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supernatants from a ConA-activated T-cell line,
designated Cl.Ly $1^+2^-/9$ and deposited at the American
Type Culture Collection (Accession Number ATCC CRL 8179)
(Nabel, G. et al., Nature, 291: 332-334 [1981]). This
5   T-cell line was derived from C57Bl/6 mice (Nabel, G. et
al., Proc. Natl. Acad. Sci. U.S.A., 78: 1157-1161
[1981]), and was maintained in modified supplemental DME
(Nabel et al., Cell, 23: 19-28 [1981]). It is a
suitable mRNA source for the polypeptides of this
10   invention, but other mammalian cell sources (including
human peripheral blood) exhibiting the appropriate
activity (e.g. MCGF or multi-CSF) may also be used.

The MC/9 cells are used to assay for MCGF
activity, preferably by a ^{3}H-thymidine incorporation
15   assay according to established methods (e.g., Nabel et
al., Nature, 291: 332-334 [1981]). Briefly, MC/9 cells
(10^4/well) are cultured in flat bottom Falcon microtiter
trays in DME supplemented with 4% fetal calf serum, 50
µM 2-mercaptoethanol (2-ME), 2mM glutamine, non-
20   essential amino acids, essential vitamins and varied
concentrations of supernatant in a final volume of 0.1
ml. To each culture is added 0.5 Ci ^{3}H-thymidine for
the last 4 hr of a 24 hr incubation period. The cells
are then harvested onto glass filters and the
25   radioactivity measured by liquid scintillation
spectrometer.

Isolation and size Fractionation of mRNA

Total cellular mRNA can be isolated by a
variety of well-known methods, e.g., by using the
30   guanidinium-thiocyanate extraction procedure of Chirgwin
et al. (Biochemistry, 18: 5294-5299 [1979]). If this
method is used, approximately 100 µg of polyA$^+$ mRNA,

selected on columns of oligo (dT) cellulose, is obtained from 1-2 x 10^8 activated helper T-cells, such as Cl.Ly $1^+2^-/9$. To fractionate the mRNA by size, 100 µg of polyA$^+$ mRNA is layered on a 10 ml 5-25% sucrose gradient (10mM Tris·HCl, pH 7.4, 100mM NaCl, 1 mM EDTA), and centrifuged for 19 hr at 26,000 rpm in a Beckman SW41 rotor. 450 µl fractions are collected and the RNA is precipitated with 2 volumes of ethanol.

Hybrid Selection and Microinjection of Xenopus laevis Oocytes

Filter hybridizations are preferably performed essentially as described by Parnes et al. (Proc. Natl. Acad. Sci. U.S.A., 78: 2253-2257 [1981]). Aliquots of eluted mRNA are injected into individual Xenopus laevis oocytes by methods well known in the art. Supernatants from viable oocytes are collected after 48 hr, pooled and assayed for activities.

Construction of cDNA Library

The cDNA library can best be constructed using the pcDV1 vector-primer and the pL1 linker fragment [available from P-L Biochemicals Inc., Milwaukee, WI] according to procedures which result in greatly enriched full-length copies of mRNA transcripts (e.g. Okayama, H. and Berg, P., Mol. Cell Biol., 2, 161-170 [1982] and Mol. Cell Biol., 3, 280-289 [1983]). The plasmid vector, which contains SV40 early promoter and SV40 RNA processing signals, is designed to promote expression of the cloned cDNA segment in mammalian cells.

Using the Okayama and Berg procedure, the cyclized vector-cDNA preparation is transformed into a competent bacterial cell, such as E. coli MC1061 cells (Casadaban, M. and Cohen, S., J. Mol. Biol., 138: 179-

207 [1980]) using calcium chloride (Cohen, S. et al.,
Proc. Nat. Acad. Sci. U.S.A., 69: 2110-2114 [1972]).
Starting with 5 µg of polyA$^+$ RNA from ConA-stimulated
Cl.Ly 1$^+$2$^-$/9 cells, about 1.5 x 10^6 independent

5 transformants are obtained. About 10^4 clones are picked
up individually and inoculated into wells of microtiter
plates (Flow Laboratories Inc., McLean, Virginia) con-
taining 200 µl of L-broth, 50 µg/ml of ampicillin, and
7% DMSO. If desired, sublibraries based on the size of

10 cDNA insert are prepared from total cDNA library as
described by Okayama, H. and Berg, P. (Mol. Cell Biol.,
3, 280-289 [1983]). Briefly, plasmid DNA is digested
with SalI, ClaI, and HindIII separately, and
electrophoresed in 1% agarose gel. After staining with

15 ethidium bromide, the gel is sliced into 7 sections
corresponding to cDNA insert sizes of 0 to 1, 1 to 2, 2
to 3, 3 to 4, 4 to 5, 5 to 6, and more than 6 kilobases
(kb). DNA is extracted from each slice, recyclized with
T4 DNA ligase, and used to transform MC1061. All

20 nucleotide sequencing can be performed according to the
procedure of Maxam, A. and Gilbert, W. (Methods
Enzymol., 65: 499-560 [1980]).

Preparation of Subtracted cDNA Probe

If desired, a ^{32}P-cDNA probe is enriched for

25 ConA-induced sequence by two cycles of cDNA absorption
in order to remove cDNA sequences common between
Cl.Ly 1$^+$2$^-$/9 and closely related, but differentiated,
cells of the immune system, such as B cell myelomas (see
Davis, M. et al., "Isolation of B4 T-Cell Specific

30 Genes", Vitteta, E. and Fox, C. eds., UCLA Symp., pg. 48
[1982]). About 2 µg of mRNA having MCGF or multi-CSF
activity from a sucrose gradient fraction is preferably
used as template for reverse transcriptase using oligo

(dT) 12-18 primers (available from Collaborative
Research, Waltham, Mass.). After hydrolysis of RNA by
alkali, ^{32}P-cDNA is hybridized with 20 µg of mRNA each
from WEHI-231, a B-cell lymphoma (see e.g. Taussig _et_
5 _al._, Immunology 39: 57-60 [1980]), and an NS-1-derived
hybridoma (ATCC accession number HB-8113) at 68°C for 14
hr (cot value=5,000). The unhybridized cDNA is
separated from cDNA/RNA hybrids by column chromatography
on hydroxyapatite. A second subtraction can then be
10 performed with unhybridized ^{32}P-cDNA using an excess of
mRNA (10 µg) from uninduced Cl.Ly 1^{+}2^{-}/9 cells as above
(cot=1,100). The single-stranded ^{32}P-cDNA enriched for
ConA-induced sequences, constituting approximately 1-2%
of the starting material, is then used for colony
15 hybridization (Maniatis, T. _et al._, "Molecular Cloning,
A Laboratory Manual", Cold Spring Harbor Laboratory,
U.S.A. [1982]).

DNA Transfections into Monkey Cells

Approximately 1x10^{6} COS-7 monkey kidney cells
20 are seeded onto 60 mm plates the day prior to
transfection. Transfections are best performed with
15 µg of plasmid DNA in 1.5 ml of DME containing 50 mM
Tris·HCl, pH 7.4, and 400 µg/ml DEAE-Dextran (Pharmacia
Fine Chemicals, Uppsala, Sweden). This solution is then
25 removed after 4 hr and replaced with 2.0 ml DME + 4%
fetal calf serum. The medium is collected after 72 hr
and assayed for MCGF or multi-CSF activity as described
above. DNA transfections may be carried out in a
variety of other cell sources as well (see below).

Multi-CSF Assays

Multi-CSF activity comprises testing for the ability to act on multipotential progenitor cells, or a number of lineage restricted cells, or both. (See generally Iscove, N. et al., J. Cell Physiol. Suppl. 1: 65-78 [1982] and Ruppert, S., Exp. Hematol. 11: 154-161 [1983].) Basically, the assay conditions allow generation of burst-forming erythroid colonies (BFU-E), granulocyte/macrophage colonies (CFU-G/M) and colonies of mixed lineages (CFU-Mixed) and are performed generally according to the procedures of Metcalf, D. et al. (J. Cell Physiol., 98: 401-420 [1979]) and Johnson, G. (J. Cell Physiol., 103: 371-383 [1980]).

CSF-c Assay (Colony Forming Unit - culture)

Bone marrow cells can be harvested from the femurs of C57B1/6 mice. The cells are washed once and a single-cell suspension prepared in Iscove's modified Dulbecco's Medium, [IMDM] (GIBCO, Grand Island, New York) + 3% Fetal Calf Serum [FCS] (GIBCO). The single-cell suspension is plated in plastic tissue-culture dishes and incubated 1-2 hours in a 37°C incubator with 6% CO_2 to allow cells to adhere to the dish. The non-adherent cells are then removed and in some cases placed over a discontinuous Percoll (Sigma Chemical Co., St. Louis, MO) gradient consisting of 2 ml layers of 40%, 50%, 60%, 70% Percoll solution (as reported by Kakiuchi et al., J. Immunol., 131: 109 [1983]). The cells at the various interfaces are harvested separately and washed twice with IMDM + 3% FCS. (Alternatively, cells not placed over Percoll can be washed once with IMDM + 3% FCS.) The separate cell pellets are then resuspended at a concentration of 4.5-6 x 10^6 cells/ml in IMDM + 15% FCS.

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CFU-c's can be assayed by using a modification of the methyl-cellulose procedure of Iscove et al. (J. Cell Physiol., 83: 309 [1974]). FCS (final concentration 25%), 2-mercaptoethanol

5   $(5 \times 10^{-5}M)$, penicillin-streptomycin (1:100 of GIBCO stocks), methyl-cellulose (1.1%, 4000 centipoise), cells $(1.5-2 \times 10^5/ml)$ and various experimental factors to be tested for CFU-c ability (30%) are mixed and 1 ml of the mixture dispensed per small petri dish. The plates are

10   incubated 7 days in a 37°C/6% CO_2 incubator. They are then scored for colonies using a dissecting microscope (4x). A colony is defined as consisting of 50 or more cells. Individual colonies can be extracted, placed on microscope slides, fixed and stained with Wright/Geims

15   (See Todd-Sanford, Clinical Diagnosis By Laboratory Methods, 15th edition, Davidsohn and Henry (eds.) 137 [1974]). Morphological analysis of cell types present per single colony is then determined.

BFU-E (Burst Forming Unit - Erythroid or CFU-E)

20   The above procedure is acceptable, with the following modifications. Either at the time of plating in methyl cellulose or 3 days later, sheep erythropoietin (Step III, Connaught Medical Research Laboratories, Philadelphia, PA) is added at a

25   concentration of 0.5-1 unit per plate. Erythroid-containing colonies (BFU-E or CFU-E) are scored after 10-14 days (from time of plating) as colonies containing visibly read elements. Individual colonies are extracted and stained as above for morphological

30   analysis.

CFU-s (Colony Forming Unit - spleen)

Bone marrow is extracted from femur bones of
C57B1/6 mice. Cells are washed twice with Dulbecco's
modified Eagle's medium [DME] (GIBCO) and either
injected immediately into the tail vein of lethally
irradiated (1000 rads) C57B1/6 recipients or treated
further. Treatment consists of the following various
procedures: 1) lysis of the cells with anti-θ and
complement, followed either by immediate injection into
recipients, or by culture for various times under
various conditions before injection; 2) alternatively,
no antiserum lysis is performed, and cells are placed
immediately into culture under various conditions. The
culture conditions can be as follows: cells are
resuspended at 1 x 10^6 cells/ml in medium consisting of
DME (GIBCO) + 2-ME (5 x 10^{-5}M), MEM-Vitamins (1:100)
(GIBCO), non-essential amino acids (1:100) (GIBCO), L-
glutamine (1:100) (GIBCO), penicillin/streptomycin
(1:100) (GIBCO), a mix of arginine, asparagine, and
folic acid, 15% FCS (GIBCO), 2mM sodium pyruvate +
various factors to be tested for maintenance of CFU-s
(final concentration 25%). This cell preparation is
then plated in 24 well tissue culture plates (Falcon) at
1 ml/well and incubated in a 37°C/10% CO_2 incubator for
various times (minimum of 7 days). Every 3-4 days, non-
adherent cells are removed, spun down, resuspended in
fresh media containing the appropriate factor, and
replated. For assays in which incubation lasted more
than 7 days, cells are "moved up" to larger plastic
tissue culture vessels in order to maintain all non-
adherent cells at a concentration not xceeding 5 x 10^5
per ml. At the end of incubation, cells are washed
twice and resuspended in DME (no suppl ments) and
injected into the tail vein of lethally-irradiated

C57B1/6 mice. Cells are injected either at specific
viable cell numbers or at specific volume fraction of
the culture. Nine to twelve days following injection,
spleens are excised and placed in Borin's fixative
5   (Mallinkrodt, St. Louis, MO). Spleen colonies are
scored as visible nodules on the spleen surface with the
aid of a dissecting microscope (4x).

Human Multi-CSF and MCGF cDNA Isolation

         DNA clones of rodent genes have been used to
10   identify and isolate DNA encoding the homologous human
genes. Because of the relatively low degree of homology
between human and rodent genes, the stringency of
hybridization conditions must be adjusted to allow for
cross-hybridization between sequences which are only 75-
15   80% homologous. Several different experimental
protocols have been used to achieve this purpose. For
example, the human C^κ immunoglobulin light chain gene
has been isolated using the corresponding mouse C^κ gene
as a probe (Heiter, P. et al., Cell 22: 197-207 [1981])
20   and mouse transplantation antigen genes have been
isolated by hybridization to DNA clones encoding their
human counterparts (Steinnetz, T. et al., Cell 24: 125-
134 [1981]).

         A preferred method entails plating γ phage
25   clones from a library of human genomic DNA (Maniatis, T.
et al., "Molecular Cloning, A Laboratory Manual", Cold
Spring Harbor Laboratory, U.S.A. [1982]) at a density of
2×10^4 to 5×10^4 plaques per 150 mm plate on an
appropriate host strain, such as E. coli LE392. Ten to
30   twenty plates are generally sufficient.

         After 10-12 hours' incubation at 37°C, the
plates are refrigerated for two hours and then a 132 mm
nitrocellulose filter is applied to the agar surface of

each plate. The filter is allowed to remain in contact
with the plate for at least five minutes, during which
time the filters are keyed to the plates by puncturing
with an ink-filled 22-gauge needle. The filters are
then peeled from the plates and incubated successively
for at least two minutes first in 250 ml of 0.1 N NaOH,
0.5 M NaCl; then in 250 ml of 0.5 M Tris·HCl pH 7.5, 1.5
M NaCl. The filters are dried on paper towels and then
baked at 80°C for 4-8 hours.

For hybridization, the filters are wetted in
1x SET (0.15 M NaCl, 30 mM Tris·HCl pH 8.0, 1 mM
Na_2EDTA), then incubated in a solution of 3x SET, 5x
Denhardt's (Denhardt, D.T., B.B.R.C. 23: 641-646
[1966]), 10% dextran sulfate, 0.1% sodium dodecyl
sulfate (SDS), and 50 µg/ml each poly (rA), poly (rC),
and poly (rG), at 65°C for 2 hrs (1.5-2 ml/filter) with
constant agitation. This solution is then discarded,
and the filters are hybridized with 0.5 µg ($> 10^8$ cpm)
of a nick-translated mouse DNA probe in the same
solution (fresh), 1.5-2 ml/filter at 65°C for 1 hour and
then at 55°C for 12-20 hours. The filters are then
washed successively in 3x SET, 1x Denhardt's; 0.1% SDS;
and 1x SET, 0.1% SDS (10-15 ml/filter) at 55°C for one
hour with gentle agitation. The filters are dried on
paper towels, then autoradiographed for 12-24 hours with
appropriate film and an intensifying screen.
Hybridizing plaques are picked from the agar plates with
sterile pasteur pipets, and each is expelled into 1 ml
of 0.1 M NaCl, 0.01 M Tris·HCl pH 7.5, 10 mm $MgCl_2$, 100
µg/ml gelatin, with 50 µl of $CHCl_3$ added. After at least
4-8 hours in the cold, the phages from each plaque are
rescreened at low density (2000-4000 plaques/150 mm
plate) by a procedure identical to that described above.

In the same manner as described previously in the mouse system, positively-hybridizing phage clones verified by re-screening can then be used as a probe to screen random colonies from a human cDNA library. The human cDNA library should be prepared using RNA from an appropriate cellular source, such as human peripheral blood T lymphocytes (see Gray, P. et al., Nature 295: 503-508 [1972]). Full length cDNA clones can be identified by expression in Cos-7 cells, again as was done for the mouse cDNA clones. The isolated human multi-CSF cDNA clones will be able to express a factor capable of stimulating human bone marrow cells.

Expression in E. coli, in Yeast and in Cell Culture

Prokaryotes, such as E. coli, are very suitable for expression of the polypeptides of the present invention (see, for example, U.S. patents number 4,338,397 and 4,411,994), provided that glycosylation is not desired. To obtain high expression levels, promoters should be utilized, such as the β-lactamase (penicillinase) and lactose promoter systems (Chang et al., Nature, 275: 615 [1978]; Itakura et al., Science, 198: 1056 [1977]; Goeddel et al., Nature 281: 544 [1979] or a tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res., 8: 4057 [1980]). These are the most commonly used promoters, but other microbial promoters are available.

Those skilled in the art will realize that not only prokaryotes but also eukaryotic microbes, such as yeast, may also be used in protein production. Saccharomyces cerevisiae is a preferred eukaryotic microorganism. Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitz man et al., J. Biol. Chem., 255: 2073

[1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7: 149 [1968]; Holland et al., Biochemistry, 17: 4900 [1978]). Other promoters that have the additional advantage of transcription controlled by growth conditions may be used. Basically any plasmid vector containing a yeast-compatible promoter, an origin of replication and termination sequences is suitable.

In addition to microorganisms, cell cultures derived from multicellular organisms (especially mammalian cells) may also be used as hosts. Examples of such useful host cell lines are HeLa cells, Chinese hamster ovary cell lines, and baby hamster kidney cell lines. Expression vectors for such cells ordinarily include, as necessary, an origin of replication, a promoter located in front of the gene to be expressed, along with any required ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. When used in mammalian cells, the expression vector often has control functions provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently SV-40. (See generally U.S.P. 4,399,216, WO 81/02425 and WO 83/03259.)

To express cDNA clones of the present invention in E. coli, suitable promoters (e.g., trp, lac, tac, λpL, etc.) and Shine-Dalgarno sequences will be fused with the entire coding sequence of those plasmids carrying an ATG codon preferably in front of the cleavage site of the signal peptide. More specifically, the cDNA clone of MCGF or multi-CSF, e.g., pcD-MCGF, is first digested with PstI and XhoI endonuclease, and about 1 kb segment containing the entire protein coding sequence is subcloned into the

appropriate E. coli expression vectors to express the
protein and signal sequence. Alternatively, in order to
express only the mature protein, the segment can be
subcloned into the PstI and SalI endonuclease sites of
5   M13mp8. Single stranded M13mp8 DNA containing the
complementary strand of protein coding sequence is
annealed with a synthetic oligonucleotide (5' GAT ACC
CAC CGT TTA 3'), and double-stranded protein coding
sequence is then synthesized by the Klenow fragment.
10   After digestion with NcoI endonuclease and treatment
with S1 nuclease, a blunt-ended DNA segment containing
the double stranded MCGF coding sequence or multi-CSF
coding sequence is inserted into an appropriate
expression vector, such as pDR540, which has the tac
15   promoter (see Russel, D.R and Bennett, G.N., Gene 20,
231-243 [1982]); and deBoer, H. et al., Proc. Natl.
Acad. Sci. U.S.A. 80, 21-25 [1983]). (See generally
Messing, J. et al., Proc. Nat. Acad. Sci. U.S.A. 74,
3642-3646 [1977]); Gronenborn, B. and Messing, J.,
20   Nature 272: 375 [1978]; Messing, J. et al., Nucl. Acid
Res. 9, 309 [1981]; and Messing, J. and Vieira, J., Gene
19, 269-276 [1982].)

To express an MCGF or multi-CSF cDNA clone in
yeast, the PstI-XhoI fragment carrying a cDNA insert is
25   isolated from pcD-MCGF plasmid, and then cloned into the
PstI-SalI sites of pUC8. The resultant plasmid B8/pUC8
is cut with PstI and digested with Bal31 to remove the
oligo (dG:dC) block placed upstream of the cDNA. An
XhoI linker is attached to Bal31-digested DNA, and the
30   plasmids are recovered in E. coli. The transformants
ar analysed to determine the size of the del tion. The
XhoI-EcoRI fragment (carrying MCGF or multi-CSF cDNA) is
then isolat d from one of the deletion derivatives,
which should have about a 20 base pair deletion, and

cloned into the <u>HindIII</u> site of pAAH5 and the <u>EcoRI</u> site
of pAAR6 by blunt-end ligation using the Klenow
fragment. (pUC8 is an M13mp7-derived system useful for
insertion mutagenesis and sequencing with synthetic

5   universal primers: See Vieira, J. and Messing, J., Gene
19: 259-268 [1982]); for pcD-X, see Okayama, H. and
Berg, P., Mol. Cell. Biol. 3: 280-289 [1983]); pAAH5 and
pAAR6 are yeast expression vectors carrying the <u>ADCI</u>
promoter and terminator: Ammer, G., "Expression of

10   Genes in Yeast using the <u>ADCI</u> promoter", Methods in
Enzymology, 101: 192-201 [1982].)

<u>Purification and Formulations</u>
       The multi-CSF and MCGF polypeptides expressed
in <u>E. coli,</u> in yeast or in other cells can be purified

15   according to standard procedures of the art, including
ammonium sulfate precipitation, fractionation column
chromatography (e.g., ion exchange, gel filtration,
electrophoresis, affinity chromatography, etc.) and
ultimately crystallization (see generally "Enzyme

20   purification and Related Techniques", Methods in
Enzymology, 22: 233-577 [1977]). Once purified,
partially or to homogeneity, the polypeptides of the
invention may be used in pharmaceutical compositions
(see below), e.g., for treating parasitic infections of

25   the gastrointestinal tract; or for research purposes,
e.g., as a supplement to hematopoietic or mast cell
media and as an antigenic substance for eliciting
specific immunoglobulins useful in immunoassays,
immunofluorescent stainings, etc. (see generally

30   "Immunological Methods", Vols. I & II, Eds. Lefkovits,
I. and Pernis, B., Academic Press, New York, N.Y. [1979
& 1981]; and "Handbook of Experimental Immunology", Ed.
Weir, D., Blackwell Scientific Publications, St. Louis,
MO [1978].)

For preparing pharmaceutical compositions
containing the polypeptides described by this invention,
these polypeptides are compounded by admixture with
preferably inert, pharmaceutically acceptable

5 carriers. Suitable carriers and processes for their
preparation are well known in the art (see e.g.
Remington's Pharmaceutical Sciences and U.S.
Pharmacopeia: National Formulary, Mack Publishing
Company, Easton, PA [1980]). The preferred course of

10 administration is parenteral and can include use of
mechanical delivery systems.

Preferably, the pharmaceutical composition is
in unit dosage form. In such form, the preparation is
subdivided into unit doses containing appropriate

15 quantities of the active component. The quantity of
active compound in a unit dose of preparation may be
varied or adjusted from 1 µg. to 100 mg., according to
the particular application and the potency of the active
ingredient. The composition can, if desired, also

20 contain other therapeutic agents.

The dosages may be varied depending upon the
requirement of the patient, the severity of the
condition being treated and the particular compound
being employed. Determination of the proper dosage for

25 a particular situation is within the skill of the art.
Generally, treatment is initiated with smaller dosages
which are less than the optimum dose of the compound.
Thereafter, the dosage is increased by small increments
until the optimum effect under the circumstances is

30 reached. For convenience, the total daily dosage may be
divided and administered in portions during the day if
desired.

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The following experimental information and data are offered by way of example and not by way of limitation.

EXPERIMENTAL

A. Cloned Inducer T Cells

1) A clone of T Cells Cl.Ly $1^+2^-/9$ (ATCC accession number CRL 8179) expressing the Thy 1^+ Ly 1^+2^- phenotype is continuously maintained at 0.5×10^5 cells/ml in Dulbecco's Modified Eagle's medium (DME) with 10% heat-inactivated fetal calf serum, 5×10^{-5} M 2-ME, 2 mM glutamine, non-essential amino acids, and essential vitamins conditioned with 25% supernatants from ConA-activated mouse Balb/c spleen cells.

2) ConA-activation of Cl.Ly $1^+2^-/9$ cells: The cells are cultured at 5×10^5/ml in DME with 4% heat-inactivated fetal calf serum, 5×10^{-5} M 2-ME, 2mM glutamine, non-essential amino acids, essential vitamins and 2 µg/ml ConA. After 12-14 hrs.' incubation at 37°C in 10% CO_2, the cell suspension is centrifuged at 1500 rpm for 10 minutes. The cell pellets are collected and frozen immediately at -70°C. The supernatants are filtered (Nalgene-0.22 microns) and stored at -20°C as a source of growth factors. Aliquots of the supernatant are assayed for MCGF activity (see below) to verify the induction of the line by the ConA treatment.

B. Cloned Mast Cells

1) A mast cell line (MC/9) (ATCC accession number CRL 8306) was cloned by limiting dilution from the

liver of a 13-day-old mouse fetus in DME with 4%
heat-inactivated fetal calf serum (FCS), 5×10^{-5} M
2-ME and 2 mM glutamine conditioned by ConA
activated Balb/c spleen cells (Nabel _et al._, Nature
5   291: 332-334 [1981]). The cell clone expresses the
Thy 1^-, Ly 1^-, 2^-, Ly 5^+ phenotype for surface
membrane glycoproteins.

2)   The mast cell clone is continuously maintained
with doubling times of 16-18 hours in DME with 10%
10   heat-inactivated FCS, 5×10^{-5} M 2-ME and 2 mM
glutamine, non-essential amino acids and essential
vitamins supplemented with 5% supernatant from ConA-
activated inducer T cell clone (see above). The
growth of the mast cell clone is dependent on the
15   active growth factor(s) obtained from the
supernatant of stimulated Cl.Ly $1^+2^-/9$ cells.

C.   Biological Assays for MCGF
1.   Tritiated Thymidine Incorporation Assay.
a) 1×10^4 MC/9 cells were cultured in flat-bottom
20   microtiter trays in 0.1 ml of DME with 4% heat-
inactivated FCS, 5×10^{-5} M 2-ME, 2 mM
glutamine, non-essential amino acids, essential
vitamins, and doubling dilutions of test
supernatant.
25   b)   The trays were incubated at 37°C in 10% CO_2.
After twenty hours, 0.5 µCi ^{3}H-thymidine (New
England Nuclear, Boston, Mass.) was added to
each culture. Four hours later, the cells were
harvested onto filter paper strips, using an
30   automated cell harvester unit. The dried
samples were dispensed into liquid scintillation
fluid and the cpm were counted in a standard β
counter.

2. Tetrazolium Salt (MTT) Colorimetric Assay.

 a) 1 x 10^4 MC/9 cells were cultured in flat-bottom microtiter trays in 0.1 ml of DME supplemented with co-factors and test supernatant as described in 1) a).

 b) The trays were incubated at 37°C in 10% CO_2. After twenty hours, 0.01 ml of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) was added to each culture. Four hours later 0.1 ml of 0.04 N HCl in isopropanol was added to each culture and thoroughly mixed. After a few minutes, the plates were read on a Dynatech MR580 Microelisa Auto Reader (Dynatech Instruments, Inc., Torrance, CA), at a wavelength of 570 nm (reference wavelength of 630nm) and a calibration setting of 1.99.

D. Isolation of mRNA from Cl.Ly 1^+2^-/9 cells.

1. Total cellular RNA was isolated from cells using the guanidine isothiocyanate procedure of Chirgwin et al. (Biochemistry, 18: 5294-5299 [1979]).

 Frozen cell pellets from uninduced or ConA-induced Cl.Ly 1^+2^-/9 were suspended in guanidine isothiocyanate lysis solution. Twenty ml of lysis solution was used for 1-2 x 10^8 cells. Pellets were resuspended by pipetting, then DNA was sheared by 4 passes through a syringe using a 16 gauge needle. The lysate was layered on top of 20 ml of 5.7 M CsCl, 10 mM EDTA in 40 ml polyallomer centrifuge tube. This solution was centrifuged at 25,000 rpm in a Beckman SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 40 hrs at 15°C. The guanidine isothiocyanate phase containing DNA was pipetted off

from the top, down to the interface. The walls of
the tube and interface were washed with 2-3 ml of
guanidine isothiocyanate lysis solution. The tube
was cut below the interface with scissors, and the
5   CsCl solution was decanted. RNA pellets were washed
twice with cold 70% ethanol. Pellets were then
resuspended in 500 μl of 10 mM Tris·HCl pH 7.4, 1 mM
EDTA, 0.05% SDS. 50 μl of 3M sodium acetate was
added and RNA was precipitated with 1 ml ethanol.
10   The RNA was collected by centrifuging and the pellets
washed once with cold ethanol.

2)   Poly A$^+$ mRNA isolation:

Washed and dried total RNA pellet was resuspended
in 900 μl of oligo (dT) elution buffer (10 mM
15   Tris·HCl, pH 7.4, 1 mM EDTA, 0.5% SDS). RNA was
heated for 3 min. at 68°C and then chilled on ice.
100 μl of 5 M NaCl was added. The RNA sample was
loaded onto a 1.0 ml oligo (dT) cellulose column
(Type 3, Collaborative Research, Waltham, MA)
20   equilibrated with binding buffer (10 mM Tris·HCl pH
7.4, 1 mM EDTA, 0.5 M NaCl, 0.5% SDS.). Flow-through
from the column was passed over the column twice
more. The column was then washed with 20 ml binding
buffer. Poly A$^+$ mRNA was collected by washing with
25   elution buffer. RNA usually eluted in the first 2 ml
of elution buffer. RNA was precipitated with 0.1
volume 3 M sodium acetate (pH 6) and two volumes of
ethanol. The RNA pellet was collected by
centrifugation, washed twice with cold ethanol, and
30   dried. The pellet was then resuspended in water.
Aliquots were diluted, and absorbance at 260 nm was
determined.

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E. Fractionation of poly A$^+$ mRNA by Sucrose Gradient
    Centrifugation:

    100 µl containing 100 µg of poly A$^+$ mRNA from D.
    2) was heated at 65°C for 1 min. and then layered
    onto a 10 ml 5-25% sucrose gradient (10 mM Tris·HCl
    pH 7.4, 100 mM NaCl, and 1 mM EDTA). The gradient
    was centrifuged in a Beckman SW41 rotor at 26,000 rpm
    for 19 hours at 5°C. 450 µl fractions were
    collected, precipitated with 2 volumes of ethanol and
    resuspended for injection into oocytes (see below).
    A parallel gradient was layered with a mixture of
    radio-labelled (^{3}H-uridine) ribosomal RNA (BRL,
    Bethesda, MA) centrifuged as described above, and 450
    µl fractions were counted in the scintillation
    counter.

    The size-fractionated Poly A$^+$ mRNA, following
    injection in Xenopus oocytes, gave a peak of MCGF
    activity by the colorimetric assay sedimenting slower
    than 18S, as shown in Figure 2. These fractions were
    enriched approximately 10-fold for MCGF mRNA and were
    utilized subsequently for the preparation of ^{32}P-
    labelled cDNA probe.

F. Oocyte Injection

    Oocytes were removed from female Xenopus laevis
    and incubated in Barth's solution (88 mM NaCl, 1 mM
    KCl, 0.33 mM Ca(NO$_3$)$_2$, 0.41 mM CaCl$_2$, 0.82 mM MgSO$_4$,
    2.4 mM NaHCO$_3$, 10 mM HEPES (pH 7.9) (Sigma Chemical
    Co., St. Louis, MO). Injection clusters of 2-3
    oocytes were prepared. RNA samples were to be
    injected dissolv d in injection buffer (40 mM
    Tris·HCl pH 7.4, 0.35 M NaCl). Total poly A$^+$ mRNA
    was resuspended at a concentration of 500 µg/ml in
    injection buffer, while RNA samples eluted from DNA

filters from hybrid selections (see below) always
contained 5 µg of calf liver tRNA as carrier and were
resuspended in 2 µl of injection buffer. 40 nl
aliquots were injected into each oocyte using
micropipets pulled by hand with tips forged using a
microforge. The pipettes were calibrated with known
volumes of sterile water. Approximately 30-40
oocytes were injected for each mRNA sample. The
injected oocytes were incubated in groups of two or
three in individual wells of 96-well microtiter
dishes containing 10 µl of Barth's solution + 1%
bovine serum albumin per oocyte. The oocytes were
kept at 19°C for 48 hours and then the supernatants
from wells containing viable oocytes were collected
and pooled. These supernatants were sterilized by
centrifuging for 10 minutes in a microcentrifuge and
then assayed for MCGF activity as described above.
Supernatants from uninjected oocytes were always
collected as a control.

The assay results from supernatants collected
from untreated or ConA-stimulated Cl.Ly $1^+2^-/9$ cells are
shown in Table I. Titration of all samples, including
the reference standard, was performed in triplicate. One
unit of MCGF is the amount of factor that results in 15%
of the maximal level of ^{3}H-thymidine incorporation
obtained using Cl.Ly $1^+2^-/9$ supernatant.

TABLE I

Cl.Ly $1^+2^-/9$-Produced MCGF Activity (units/ml)

	Cell Supernatant	Injected mRNA
without ConA	50	<40
with ConA	26,383	1,403

G.   cDNA Library Construction:

1)   Preparation of vector primer and oligo dG-tailed
          linker DNAs:

The procedure of Okayama & Berg (Mol. & Cell.
Biol. 2: 161-170 [1982]) was used with only minor
modifications and adapted to the pcDV1 and pL1
plasmids described by Okayama & Berg [Mol. & Cell.
Biol. 3: 380-389 (1983)].

An 80 μg sample of pcDV1 DNA was digested at 30°C
with 20 U of KpnI endonuclease in a reaction mixture
of 450 μl containing 6 mM Tris·HCl (pH 7.5), 6 mM
$MgCl_2$, 6 mM NaCl, 6 mM 2-ME, and 0.1 mg of bovine
serum albumin (BSA) per ml. After 16 hr the
digestion was terminated with 40 μl of 0.25 M EDTA
(pH 8.0) and 20 μl of 10% sodium dodecyl sulfate
(SDS); the DNA was recovered after extraction with
water-saturated 1:1 phenol-$CHCl_3$ (hereafter referred
to as phenol-$CHCl_3$) and ethanol precipitation.
Homopolymer tails averaging 60, but not more than 80,
deoxythymidylate (dT) residues per end were added to
the KpnI endonuclease-generated termini with calf
thymus terminal transferase as follows: The reaction
mixture (38 μl) contained sodium cacodylate-30 mM
Tris·HCl pH 6.8 as buffer, with 1 mM $CoCl_2$, 0.1 mM
dithiothreitol, 0.25 mM dTTP, the KpnI endonuclease-
digested DNA, and 68 U of the terminal
deoxynucleotidyl transferase (P-L Biochemicals, Inc.,
Milwaukee, WI). After 30 min. at 37°C the reaction
was stopped with 20 μl of 0.25 M EDTA (pH 8.0) and 10
μl of 10% SDS, and the DNA was recovered after
several extractions with phenol-$CHCl_3$ by ethanol

precipitation. The DNA was then digested with 15 U
of EcoRI endonuclease in 50 μl containing 10 mM
Tris·HCl pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol,
and 0.1 mg of BSA per ml for 5 hr at 37°C. The large
fragment, containing the SV40 polyadenylation site
and the pBR322 origin of replication and ampicillin-
resistance gene, was purified by agarose (1%) gel
electrophoresis and recovered from the gel by a
modification of the glass powder method (Vogelstein,
B. & Gillespie, D., Proc. Nat. Acad. Sci. 76: 615-619
[1979]). The dT-tailed DNA was further purified by
absorption and elution from an oligo (dA)-cellulose
column as follows: The DNA was dissolved in 1 ml of
10 mM Tris·HCl pH 7.3 buffer containing 1 mM EDTA and
1 M NaCl, cooled at 0°C, and applied to an oligo
(dA)-cellulose column (0.6 by 2.5 cm) equilibrated
with the same buffer at 0°C. The column was washed
with the same buffer at 0°C and eluted with water at
room temperature. The eluted DNA was precipitated
with ethanol and dissolved in 10 mM Tris·HCl pH 7.3
with 1 mM EDTA.

The oligo (dG) tailed linker DNA was prepared by
digesting 75 μg of pL1 DNA with 20 U of PstI
endonuclease in 450 μl containing 6 mM Tris·HCl pH
7.4, 6 mM $MgCl_2$, 6 mM 2-ME, 50 mM NaCl, and 0.01 mg
of BSA per ml. After 16 hr at 30°C the reaction
mixture was extracted with phenol-$CHCl_3$ and the DNA
was precipitated with alcohol. Tails of 10 to 15
deoxyguanylate (dG) residues were then added per end
with 46 U of terminal deoxynucleotidyl transferase in
the same reaction mixture (38 μl) as described above,
except that 0.1 mM dGTP replaced dTTP. After 20 min.
at 37°C the mixture was extracted with phenol-$CHCl_3$,
and after the DNA was precipitated with thanol it
was digested with 35 U of HindIII endonuclease in

50 μl containing 20 mM Tris·HCl pH 7.4, 7 mM MgCl$_2$, 60 mM NaCl, and 0.1 mg of BSA at 37°C for 4 hr. The small oligo (dG)-tailed linker DNA was purified by agarose gel (1.8%) electrophoresis and recovered as described above.

2) cDNA Library Preparation:

Step 1. cDNA synthesis.

The reaction mixture (10 μl) contained 50 mM Tris·HCl pH 8.3, 8 mM MgCl$_2$, 30 mM KCl, 0.3 mM dithiothreitol, 2 mM each dATP, dTTP, dGTP, and dCTP, 20 μCi ^{32}P-dCTP (3000 Ci/mmole), 2 μg polyA$^+$ RNA from Con-A induced Cl.Ly 1$^+$2$^-$/9, 60 units RNase (Biotec, Inc., Madison, WI), and 2 μg of the vector-primer DNA (15 pmol of primer end), and 45 U of reverse transcriptase. The reaction was incubated 60 min at 42°C and then stopped by the addition of 1 μl of 0.25 M ETDA (pH 8.0) and 0.5 μl of 10% SDS; 40 μl of phenol-CHCl$_3$ was added, and the solution was blended vigorously in a Vortex mixer and then centrifuged. After adding 40 μl of 4 M ammonium acetate and 160 μl of ethanol to the aqueous phase, the solution was chilled with dry ice for 15 min., warmed to room temperature with gentle shaking to dissolve unreacted deoxynucleoside triphosphates that had precipitated during chilling, and centrifuged for 10 min. in an Eppendorf microfuge. The pellet was dissolved in 10 μl of 10mM Tris·HCl pH 7.3 and 1 mM EDTA, mixed with 10 μl of 4 M ammonium acetate, and reprecipitated with 40 μl of ethanol, a procedure which removes more than 99% of unreacted deoxynucleoside triphosphat s. The pellet was rinsed with ethanol.

Step 2: Oligodeoxycytidylate [oligo (dC)]
addition.

The pellet containing the plasmid-cDNA:mRNA was
dissolved in 20 µl of 140 mM sodium cacodylate-30 mM
Tris·HCl pH 6.8 buffer containing 1 mM $CoCl_2$, 0.1 mM
dithiothreitol, 0.2 µg of poly(A), 70 µM dCTP, 5 µCi
^{32}P-dCTP, 3000 Ci/mmole, and 60 U of terminal
deoxynucleotidyl transferase. The reaction was
carried out at 37°C for 5 min. to permit the addition
of 10 to 15 residues of dCMP per end and then
terminated with 2 µl of 0.25 M EDTA (pH 8.0) and 1 µl
of 10% SDS. After extraction with 20 µl of phenol-
$CHCl_3$, the aqueous phase was mixed with 20 µl of 4 M
ammonium acetate, the DNA was precipitated and
reprecipitated with 80 µl of ethanol, and the final
pellet was rinsed with ethanol.

Step 3: HindIII endonuclease digestion.

The pellet was dissolved in 30 µl of buffer
containing 20 mM Tris·HCl pH 7.4, 7 mM $MgCl_2$, 60 mM
NaCl, and 0.1 mg of BSA per ml and then digested
with 10 U of HindIII endonuclease for 2 hr at
37°C. The reaction was terminated with 3 µl of
0.25 M EDTA (pH 8.0) and 1.5 µl of 10% SDS and,
after extraction with phenol-$CHCl_3$ followed by the
addition of 30 µl of 4 M ammonium acetate, the DNA
was precipitated with 120 µl of ethanol. The
pellet was rinsed with ethanol and then dissolved
in 10 µl of 10 mM Tris·HCl (pH 7.3) and 1 mM EDTA,
and 3 µl of ethanol was added to prevent freezing
during storage at -20°C.

Step 4: Cyclization mediated by the oligo
(dG)-tailed linker DNA.

A 9 µl sample of the HindIII endonuclease-digested
oligo (dC)-tailed cDNA:mRNA plasmid (90% of the

sample) was incubated in a mixture (90 μl) contain-
ing 10 mM Tris·HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl,
and 1.8 pmol of the oligo (dG)-tailed linker DNA at
65°C for 5 min, shifted to 42°C for 60 min, and

5   then cooled to 0°C. The mixture (90 μl) was ad-
justed to a volume of 900 μl containing 20 mM
Tris·HCl pH 7.5, 4 mM MgCl$_2$, 10 mM (NH$_4$)$_2$SO$_4$, 0.1 M
KCl, 50 μg of BSA per ml, and 0.1 mM β-NAD; 6 μg of
E. coli DNA ligase were added and the solution was

10   then incubated overnight at 12°C.
        Step 5: Replacement of RNA strand by DNA.
To replace the RNA strand of the insert, the ligation
mixture was adjusted to contain 40 μM of each of the
four deoxynucleoside triphosphates, 0.15 mM β-NAD, 4

15   μg of additional E. coli DNA ligase, 16 U of E. coli
DNA polymerase I (PolI,) and 9 U of E. coli RNase
H. This mixture (960 μl) was incubated successively
at 12°C and room temperature for 1 hr each to promote
optimal repair synthesis and nick translation by

20   PolI.
        Step 6: Transformation of E. coli.
Transformation was carried out using minor
modifications of the procedure described by Cohen et
al. (Proc. Nat. Acad. Sci. U.S.A., 69: 2110-2114

25   [1972]). E. coli K-12 strain MC1061 (Casadaban, M.
and Cohen, S., J. Mol. Biol. 138: 179-207 [1980]) was
grown to 0.5 absorbancy unit at 600 nm at 37°C in 20
ml of L-broth. The cells were collected by
centrifugation, suspended in 10 ml of 10 mM Tris·HCl

30   pH 7.3 containing 50 mM CaCl$_2$, and centrifuged at 0°C
for 5 min. The cells were resuspended in 2 ml of the
above buffer and incubated again at 0°C for 5 min.;
then, 0.2 ml of the cell suspensions was mixed with
0.1 ml of the DNA solution (step 5) and incubated at

0°C for 15 min. Next the cells were kept at 37°C for 2 min. and thereafter at room temperature for 10 min.; then 0.5 ml of L-broth was added, and the culture was incubated at 37°C for 30 min., mixed with
5   2.5 ml of L-broth soft agar at 42°C, and spread over L-broth agar containing 50 μg of ampicillin per ml. After incubation at 37°C for 12 to 24 hr, individual colonies were picked with sterile tooth-picks.

Approximately 1×10^6 independent cDNA clones
10   were generated and, of these, 10,000 clones were picked up individually and inoculated into wells of microtiter plates containing 200 μl of L-broth, 50 μg/ml ampicillin, and 7% DMSO. Random pools of approximately 1000 clones each were generated, and
15   plasmid DNA prepared for hybrid selection experiments.

H. Hybrid selections.

Hybrid selections were performed with eight cDNA plasmid preparations, taken from the random pools
20   described above.

1) Preparation of DNA filters

All plasmid DNAs were linearized by digestion with ClaI prior to binding to nitrocellulose filters. Digestions were performed in 50 μl
25   containing: 10 mM Tris·HCl pH 7.9, 10 mM MgCl₂, 10 μg plasmid DNA, 50 mM NaCl, and 10 units ClaI. Following a 2 hr incubation at 37°C, samples were diluted to 200 μl with TE (10 mM Tris·HCl pH 8.0, 1 mM EDTA) and extracted with an equal volume (200 μl)
30   of phenol saturated with TE. 20 μl of 3M sodium acetate (pH 6) was added to the aqueous phase, and this was precipitated with 2 volumes of ethanol. The DNA pellets were recovered by centrifugation and then

washed with 70% ethanol. The dried pellet was resuspended in 150 μl of sterile water for each 10 μl of DNA. Duplicate filters were prepared for each DNA sample, 10 μg DNA per filter. The DNA in 150 μl of water was boiled for 10 min, then 150 μl 1N NaOH was added and the solution incubated 20 min at room temperature. The sample was chilled on ice, then 150 μl 1M HCl, 1M NaCl, 0.3M Na-citrate and 0.5M Tris·HCl pH 8.0 was added.

0.9 cm Millipore HAWP filters wet with distilled water were fitted into Schleicher-and-Schuell microfiltration apparatus. The denatured and neutralized DNA solution from above was filtered through by centrifugation at 500 rpm for 5 min. Filters were washed with 1 ml of 6xSSC (0.15 M NaCl, 0.015 M Na citrate) and then air-dried before baking 2 hrs. at 80°C.

2) Hybridizations

Hybridizations were performed in 200 μl containing 65% (v/v) redistilled formamide, 20 mM PIPES, pH 6.4, 0.4 M NaCl, 200 μg/ml calf liver tRNA, and 100 μg/ml polyA$^+$ mRNA from ConA-induced Cl.Ly 1$^+$2$^-$/9. Each hybridization solution was heated for 3 min at 70°C and then two DNA filters (10 μg DNA/filter) were cut into quarters and added to the solution. Hybrids were incubated at 50°C for 4 hours followed by 4 hour incubations at 46° and 42°C. After this period the supernatants were removed and the filters washed 3 times with 1 ml of: 10 mM Tris·HCl pH 7.4, 0.15M NaCl, 1 mM EDTA, 0.5% SDS. This was followed by three 1 ml washes with the same buffer lacking SDS. Both buffers were kept at 65° for the washes. To elute the hybridized mRNA, 400 μl of distilled water with 5 μg calf liver tRNA was

added to the vial with the filters. The tubes were
boiled for 3 min and then quick chilled in dry
ice/ethanol. Samples were thawed and the eluted RNA
precipitated with 2 volumes of ethanol and 0.1 volume

5   3M Na acetate (pH 6). RNA pellets were collected by
centrifugation and washed twice with 70% ethanol.
The dried pellets were resuspended in 2 μl of oocyte
injection buffer and the entire sample was injected
into oocytes (see above).

10   Of 8 initial pools which were screened in this
manner, several were positive, and one pool showing
the highest level of MCGF activity was chosen for
further analysis. This pool, which consisted of 672
individual clones, was subdivided further into 14

15   sub-pools of 48 clones each. Plasmid DNA from these
sub-pools was used in a second series of hybrid
selections. Only one of these sub-pools gave a
positive signal. The 48 clones were then screened
with the two subtracted cDNA probes as described

20   below.

I.   Preparation of Subtracted cDNA Probe
1) ^{32}P-cDNA synthesis:
     2 μg of polyA$^+$ mRNA from the MCGF peak fraction
of the sucrose gradient from above was resuspended in

25   2 μl of water. This was heated for 5 min at 65°C,
then added to a reaction containing 50 mM Tris·HCl pH
8.3, 8 mM MgCl$_2$, 30 mM KCl, 0.7 mM DTT, 1 mM each of
dATP, dGTP and dTTP, 34 μM dCTP, 10 μg/ml oligo-[12-
18]-(dT) (Collaborative Research), 100 μg/ml

30   Actinomycin D, 500 μCi α^{32}P-dCTP (Amersham, 3000
Ci/mmole) and 150 units reverse transcriptase (Life
Sciences, Inc., St. Petersburg, FL) in a total volume
of 100 μl. Following a 2 hr incubation at 40°C, 0.5

µl of the reaction was removed for precipitation in trichloroacetic acid to determine the amount of ^{32}P incorporated. Then, 100 µl of 0.2 N NaOH was added, and the sample was heated 20 min at 70°C to hydrolyze the RNA. After cooling, the reaction was neutralized with 20 µl of 1 N HCl, and 4 µl of 1 mg/ml tRNA was added as carrier. The sample was extracted twice with an equal volume of phenol-chloroform (1:1). It was then precipitated with an equal volume of 4 M ammonium acetate and 2 volumes of ethanol. The pellet was resuspended in 100 µl water, the precipitation repeated, and the pellet washed twice with 80% ethanol.

2) First subtractive hybridization:

^{32}P-cDNA (synthesized as described above) was co-precipitated with 20 µg of polyA$^+$ mRNA from WEHI-3 and 20 µl of poly A$^+$ mRNA from a B-cell hybridoma. The pellet was resuspended in 7 µl water, 1 µl 4 M Na phosphate pH 7, 0.1 µl 20% SDS, and 0.1 µl 0.1M EDTA, and then the entire sample was sealed in a capillary tube. The sample was heated 30 min at 90°C, then shifted to 68°C for 14 hrs (Cot = 5000). The hybridization mixture was then diluted to 1 ml with 0.12 M sodium phosphate pH 7.0 and 0.1% SDS, and the temperature of the mixture raised to 60°C. The mixture was then loaded on a column of 0.4 gm hydroxyapatite equili- brated in the same buffer and kept at 60°C. The flowthrough was collected and the column was then washed with 5 ml of the same buffer at 60°C. 1 ml fractions were collected and 1 µl aliquots of each fraction were counted in a scintillation counter. The peak of single stranded cDNA in fractions 2, 3, and 4 was pooled. This material, representing

66.5% of the starting ^{32}P-cDNA, was concentrated to 0.4 ml by extraction with 2-butanol and then desalted by chromatography on a 2 ml Sephadex G-25 column.

5   3)   Second subtractive hybridization:

The desalted sample from above was concentrated by ethanol precipitation and then co-precipitated with 9.5 μg of poly A$^+$ mRNA from uninduced Cl.Ly 1$^+$2$^-$/9. The washed and dried pellet was

10   resuspended in 10 μl water, 1.5 μl 4M sodium phosphate pH 7, 0.15 μl 20% SDS and 0.15 μl 0.1 M EDTA. The sample was incubated in a sealed capillary tube for 30 min at 90°C and then at 68°C for 20 hr. Chromatography on hydroxyapatite was

15   repeated as described above. The single stranded cDNA which eluted from the column at 60°C represented 17% of the starting material. This ^{32}P-cDNA was used for colony hybridizations of the 48 colonies in the sub-pool identified by hybrid

20   selections. Three colonies hybridized with the probe and were used for further hybrid selection. One of these, designated clone 5G, was reproducibly positive.

J.   Size fractionated sub-library

25   30 μg of plasmid DNA representing the entire cDNA library (pcD-X DNA) was digested separately with the restriction enzymes SalI, HindIII, and ClaI to linearize the plasmid. The restricted DNAs were size-fractionated on a 1% agarose gel to

30   separate plasmids having different size cDNA inserts. Segments were excised from the gel representing plasmids with cDNA inserts of the following size ranges:

0 - 1kb

1 - 2kb

3 - 4kb

4 - 5kb

5 - 6kb

6 kb and longer

DNA was eluted from each gel slice using the glass powder method of Vogelstein and Gillespie (Proc. Nat. Acad. Sci. U.S.A., 76: 615-619 [1970]). The eluted DNAs from the 3 digests were pooled on the basis of size, and treated with T4 ligase to recyclize in a total volume of 15 µl containing 50 mM Tris·HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP and 100 µg/ml BSA. The ligation reactions were incubated 16 hr at 12°C. 3 µl of each combined size fraction was used to transform E. coli strain MC 1061 using the method of Cohen et al. (Proc. Nat. Acad. Sci. U.S.A., 69: 2110-2114 [1972]). A library of 1.1 x 10^5 independent transformations was obtained for the fraction containing cDNA inserts 1-2 kb in length, which was used for subsequent screening for full-length MCGF clones.

K. Screening of Size-fractionated Sub-library

Preliminary restriction endonuclease analysis, as well as DNA sequence data, indicated that the cDNA insert for clone 5G (identified by hybrid selection) was approximately 650 base pairs long. An internal BamH1-NcoI restriction fragment was isolated from clone 5G. The fragment was

dephosphorylated with calf intestinal alkaline phosphatase according to the method of Chacomas, G. and Sande, J. (Methods Enzymol. 65: 75-79 [1980]). The fragment was then labelled using γ^{32}P-ATP and T4 polynucleotide kinase according to the method of Maxam, A. and Gilbert, W. (Methods Enzymol. 65: 499-507 [1980]). This labelled fragment was then used to probe an RNA blot of ConA-induced Cl.Ly 1$^+$2$^-$/9 mRNA. A single band, approximately 1 kb long, was detected, suggesting that clone 5G was not a full-length clone.

We therefore used the same probe from the 5G cDNA insert to screen the sub-library enriched for 1-2 kb inserts (from above). We employed the method of Hanahan and Moselson (Gene, 10: 63 [1980]) as described by Maniatis, T. et al. (Molecular Chem., Cold Spring Harbor Laboratory [1982]). Approximately 500-1000 bacteria were spread on 80 mm nitrocellulose filters and incubated on L-broth plates containing 50 μl/ml ampicillin at 37°C, and the bacterial colonies transferred to a second nitrocellulose filter. The duplicate filters were incubated on L-broth + ampicillin for 8 hrs. at 37°C, transferred to L-broth plates containing 10 μg/ml chloramphenicol and incubated overnight to amplify the copy number of the plasmid. The DNA from the colonies was bound to the nitrocellulose following lysis of the colonies with SDS, denaturation with NaOH and neutralization as described by Maniatis, T. et al., supra.

Approximately 20,000 colonies were screened and 19 colonies were reproducibly positive following rescreening with the probe. Plasmid DNA from these

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colonies was prepared and used in transfection
experiments.

L.   DNA Transfections
      One day prior to transfection, approximately 10^6
5   COS-7 monkey cells were seeded onto individual 60
     mm plates in DME containing 10% fetal calf serum
     and 2 M glutamine. To perform the transfection,
     the medium was aspirated from each plate and
     replaced with 1.5 ml of DME containing 50 mM
10   Tris·HCl pH 7.4, 400 µg/ml DEAE-Dextran and 15 µg
     of the plasmid DNAs to be tested. The plates were
     incubated for four hours at 37°C, then the DNA-
     containing medium was removed, and the plates were
     washed twice with 2 ml of serum-free DME. 2.0 ml
15   of DME containing 4% fetal calf serum and 2 mM
     glutamine was added to the plates, which were then
     incubated 72 hours at 37°C. The growth medium was
     collected and assayed for MCGF activity as
     described above.
20   Five of the six initial positive clones were
     examined by transfection and the results are shown
     in Table II. Mock infected COS-7 cells were
     treated identically, but omitting DNA.

TABLE II
Transient Expression of MCGF in Monkey Cells

Clone	cDNA Start Point*	Length of Oligo(dG) Block***	MCGF Activity in units/ml
Mock	–	–	<20
B4	41	13	5,228
B5	ND**	ND	7,371
B6	1	13	3,307
B8	1	13	6,929
B9	1	13	3,362
Cl.Ly 1^+2^-/9	–	–	19,769

*   The 5' end of MCGF cDNA expressed as nucleotide
    residue in Fig. 1.

** Not determined; cDNA start point is located at the 5'
    side of position 41.

*** Oligo (dG) block at the 5' end of MCGF cDNA.

A plasmid (pcD-MCGF) carrying a full-length
MCGF and multi-CSF (see below) cDNA insert is shown in
Figure 3, and an E. coli bacterium carrying the plasmid
has been deposited with the ATCC (accession number
39467). The 950 bp insert is contained in the pcD
expression vector. Transcription from the SV40 early
promoter is indicated by the arrow. The location of
the splice donor and acceptor sites are shown. The
polyadenylation signal, also derived from SV40, is
located at the 3' end of the cDNA insert. The cDNA
insert is heavily shaded. The remainder of th vector
sequences are derived from pBR322 including the β-
lactamase gene (Amp^R) and the origin of replication.

Figure 4 shows the restriction endonuclease cleavage
map of the cDNA insert of the present invention, and
Figure 1 contains the nucleotide sequence and putative
amino acid sequence.

5   Three cDNA inserts contain a single open
reading frame consisting of 166 codons beginning with
the methionine codon at position 28. In addition to
this putative initiation codon, two other methionine
codons occur, 12 and 18 codons downstream from the
10   first. A fourth cDNA clone starts 40 base pairs
downstream from the 5' ends of the other three
inserts. This shorter cDNA clone lacks the first
methionine codon but still makes active MCGF upon
introduction into COS cells. Thus, one of the two ATG
15   codons downstream can apparently serve as the
initiation codon.

          Clone B9 expressed in COS-7 cells (COS-MCGF)
was used to evaluate, in the absence of other T cell
products, its spectrum of activities. The expressed
20   material is not mitogenic for T or B cells, fails to
induce immunoglobulin production by B cells (Table
III), and does not induce macrophage Ia expression.
However, this gene product does stimulate the formation
of hematopoietic colonies in bone marrow cells
25   suspended in methylcellulose, demonstrating that a
single gene product can exhibit both MCGF and colony
stimulating activities.

TABLE III. COS-MCGF stimulates BFU-E, CFU-C and CFU-mixed
in methylcellulose bone marrow cultures

Supernatant added to culture	Expt.	Non-erythroid CFU-C*	BFU-E*	CFU-Mixed*	TCGF** Activity Units/ml	BCGF† Activity Units/ml	PFC†† (10⁶ B cells)
COS-7 cells transfected with MCGF cDNA	1	263 ± 25.5	5.3 ± 1.7	12.7 ± 1.2	0	0	40
	2	224 ± 17.6	3.3 ± 2	4.3 ± 1.1			
MOCK transfected COS-7 cells	1	0	0	0	0	0	33
	2	1.7 ± 0.6	0	0			
L cells	1	304 ± 25.2	0	0	ND	ND	ND
Cl.Ly 1+2−/9	1	404 ± 23.0	5.0 ± 1.2	18.0 ± 2.1	3,250	512	4,560
Medium	1	0	0	0	0	0	6

ND = not determined.   *, **, †, †† : For these footnotes, see next page.

Notes from Table III

* Number of colonies per 1.5×10^5 bone marrow cells.
  Each value represents mean ± SEM of colonies
  determined from triplicate cultures. 1.5×10^5 non-
  adherent, light density (<1.077 g/ml) bone marrow
  cells from C57Bl/6 mice were suspended in 1 ml
  aliquots in 35 mm dishes containing 0.9% methyl-
  cellulose, 20% FCS, Iscove's modified Dulbecco's
  medium, 50 μM 2-ME, and 30% medium conditioned by
  cell supernatants as indicated. Mouse L cells were
  used as a source of CSF, which induces the formation
  of macrophage colonies. Following 5 days of
  incubation at 37°C in CO_2, 0.5 - 1.0 unit of
  erythropoietin (Connaught step III) was added to each
  plate. After incubating the plates for an additional
  7 days, the colonies were counted using a dissecting
  microscope.

** TCGF activity was assayed as previously described.
   One unit of TCGF activity is defined as that amount
   which causes 25% of the maximum level of ^{3}H-thymidine
   incorporation in 5×10^3 HT-2 cells.

† B cells were purified from spleen cells of C57Bl/6
  mice and assayed for proliferation as described. One
  unit of BCGF activity is defined as that amount which
  causes 50% of the level of ^{3}H-thymidine incorporation
  in 1×10^5 B cells stimulated by 2
  μg/ml LPS.

†† Total immunoglobulin-secreting plaque-forming cells
   were enumerated by a modification of the hemolytic
   plaque assay.

Specifically, the expressed B-9 clone was tested under conditions which allow generation of BFU-E, CFU-G/M and CFU-Mixed. Table III shows that three types of colonies could be identified and enumerated in cultures of bone marrow cells incubated with the expressed material. The most prevalent type consisted of colorless colonies lacking hemoglobinized elements. Their morphology was typical of granulocyte/macrophage colonies, the existence of which was later confirmed by histochemical staining. Also present were some large macroscopic colonies containing a multicentric arrangement of uniformly red cell clusters, which were designated BFU-E. We further observed a few colonies containing hemoglobinized cells mixed with large and small translucent cells, which were counted as mixed.

The composition of these various colonies was analyzed by applying selected colonies to glass slides and staining with Wright-Giemsa or nonspecific esterase stains. Over 300 colonies were examined. The majority of these colonies (89%) were composed of granulocytes, macrophages or a granulocyte/macrophage mixture, while four percent consisted of mast cells. The remainder were composed of mixed lineages other than neutrophil/macrophage. Differential counts of 10 representative mixed colonies compiled from several experiments are presented in Table IV. The presence of several cell types within single colonies suggests that these colonies derive from pluripotent progenitor cells.

Table IV.
Cellular composition of mixed hematopoietic colonies picked
from bone marrow cultures grown in COS-MCGF conditioned medium

| Colony Number | .... *Differential Counts (%)............ | | | | | | |
	E	n	m	e	mast	M	Bl
1	21				76		3
2		36	35	20	9		
3	97		1			2	
4			22		78		
5	17		74		9		
6		64	15	21			
7	18	81				1	
8	27	40		33			
9		86		14			
10	96					3	1

* Differential counts of greater than 200 nucleated cells/colony. Abbreviations used are: E, erythrocyte; n, neutrophil; m, macrophage/monocyte; e, eosinophil; mast, mast cell; M, megakaryocyte; and Bl, blast cell.

The effects of the expressed B-9 clone were assessed on early uncommitted stem cells according to a CFU-S assay of Till and McCullock (Radiat. Res., 14: 213-222 [1961]), as modified by Schrader, J., and Clark-Lewis, I. (J. of Immunol., 129: 30-35 [1982]). When non-adherent bone marrow cells, depleted of T cells, were incubated for one we k in COS-MCGF medium and injected by vein into lethally irradiated mice, macroscopic colonies appeared in the spleen (Table V).

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In contrast, cells incubated in supernatants of mock transfected COS-7 cells formed no colonies.

TABLE V.
Detection of CFU-S in bone marrow cells cultured
for one week in COS-MCGF conditioned medium.

Supernatant added to culture	Expt. No.	Spleen nodules/ mouse*	CFU-S**
COS-7 cells transfected	1	8.8 ± 1.5	264
with full-length MCGF cDNA	2	6.2 ± 2.8	186
COS-7 cells transfected with incomplete MCGF cDNA†	2	0.8 ± 0.7	24
Mock transfected COS-7 cells	1	0.8 ± 0.7	24
	2	0.7 ± 0.6	21
L cells	1	0.3 ± 0.5	9
	2	Not done	—
Medium	1	0.3 ± 0.5	9
	2	0.5 ± 0.5	15

* The mean ± SEM of spleen colonies detected in 5 individual mice.

** The mean of CFU-S calculated to reflect the frequency of CFU-S in the total culture of 3×10^6 bone marrow cells.

† Incomplete MCGF cDNA clone lacks the coding region for the NH_2-terminal 55 amino acids.

Light density (< 1.077) C57B1/6 bone marrow cells treated with anti-Thy1 antibody and complement were plated at 1×10^6 cells/ml

in Iscove's modified Dulbecco's medium supplemented with 20% fetal calf serum, 50 ₥M 2-ME and 30% conditioned medium, as indicated above. Non-adherent cells were removed three times during the one-week incubation period and replated in fresh medium. The cells were harvested after one week, washed twice and diluted to the original culture volume in phosphate-buffered saline. Each lethally-irradiated (1,000 R) C57B1/6 recipient was injected i.v. with 0.1 ml of the cell suspension. After 9 days, the spleens were removed and the spleen nodules were counted using a dissecting microscope.

To summarize, in addition to the mast cell growth factor activity characterized initially, COS-MCGF has erythroid burst-promoting activity and allows expansion of stem cells and early committed progenitor cells of several lineages, including monocytic/granulocytic, erythroid and megakaryocytic cells. This range of activities indicates that the cDNA clones of the present invention encode proteins having the characteristics of growth factors for hematopoietic cells for multiple lineages.

From the foregoing, it will be appreciated that the cDNA clones of the present invention provide accurate and complete sequence data on mammalian multi-CSF and mast cell growth factors. The invention also provides to those skilled in the art means for producing significant quantities of such factors (essentially free from other hematopoietic factors) for the improved in vitro maintenance of mast cells and other hematopoietic cells. Further, the information gleaned from the cDNA clones increases understanding of the mammalian immune response, nhancing therapeutic potentialities.

CLAIMS:

1. A process for producing a polypeptide exhibiting
mammalian multi-lineage cellular growth factor activity
and/or mammalian mast cell growth factor activity, said
process comprising the steps of:

5

    a)   providing a vector comprising a
nucleotide sequence coding for said
polypeptide, especially a cDNA sequence
derived from an mRNA sequence coding for
said polypeptide, wherein the nucleotide
sequence is capable of being expressed
by a host containing the vector;

10

    b)   incorporating the vector into the host;
and

    c)   maintaining the host containing the
vector under conditions suitable for
transcription of the nucleotide sequence
into said polypeptide.

15

2. A process as claimed in claim 1 wherein the host is
a mammalian cell transformed or transfected with the
vector, in particular wherein said polypeptide is
glycosylated.

20

3. A process as claimed in claim 1 or claim 2 wherein
the vector comprises the nucleotide coding for said
polypeptide linked to a second nucleotide sequence, and
this second nucleotide sequence comprises a promoter
sequence which promotes expr ssion of the nucleotide
sequence coding for said polypeptide, in particular an
SV40 virus early r gion promot r and an SV40 virus late
region polyadenylation. sequence.

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4. A process as claimed in any of claims 1 to 3, wherein the nucleotide sequence codes for a polypeptide having hematopoietic cell growth activity and in particular is characterized by one or more of the following features:

5   (a) the nucleotide sequence coding for said polypeptide has substantially the sequence shown in Figure 1;

               (b) the nucleotide sequence coding for said polypeptide is different from, but is

10   capable of hybridizing with, the nucleotide sequence shown in Figure 1;

               (c) the nucleotide sequence includes a portion coding for at least a part of a leader sequence of the polypeptide.

15   5. A polypeptide consisting essentially of at least a substantial portion of the amino acid sequence shown in Figure 1 and exhibiting mammalian multi-lineage growth factor activity and/or mammalian mast cell growth factor activity, especially such activity on a

20   hematopoietic cell line, e.g. such activity on mouse cells, in particular said polypeptide in substantially pure form and essentially free from other mammalian hematopoietic cell proteins.

6. A nucleic acid sequence that codes either for a

25   polypeptide exhibiting mouse multi-lineage cellular growth factor activity and/or mast cell growth factor activity and is capable of hybridizing to a second nucleic acid sequence coding for another mammalian cellular growth factor, in particular a DNA sequence

30   coding for at 1 ast a portion of the polypeptide of

Figure 1; or for a polypeptide exhibiting mammalian multi-lineage cellular growth factor activity and/or mast cell growth factor activity and capable of hybridizing to a second nucleic acid sequence coding for a mouse cellular growth factor.

7. A vector consisting essentially of the DNA sequence of claim 6, in particular a replicable vector capable of expressing a DNA sequence of claim 6, when said vector is incorporated into a microorganism or cell.

8. A microorganism or cell transformed or transfected with the replicable expression vector of claim 7.

9. A pharmaceutical composition consisting essentially of a polypeptide having mammalian multi-lineage growth factor activity and/or mammalian mast cell growth factor activity and a therapeutically compatible carrier.

10. A process for enhancing cell growth comprising contacting said cell preferably in vitro with a polypeptide having a substantial portion of the amino acid sequence of Figure 1.

11. A process for preparing a polypeptide exhibiting mammalian multi-lineage cellular growth factor activity, which comprises cultivating, in an aqueous nutrient medium, a prokaryotic microorganism or eukaryotic cell which has been transfected or transformed with a vector comprising a substantial portion of the DNA sequence shown in Figure 1.

12. A transformed organism or cell which contains at least a portion of a gene or other DNA sequence coding

for one or more polypeptides having mammalian multi-lineage cellular growth factor activity and/or mammalian mast cell growth factor activity.

13. A recombinant DNA molecule consisting of segments of DNA from different genomes which have been joined end to end outside of living cells and have the capacity to infect some host and to be maintained therein, and the progeny thereof, comprising a DNA sequence selected from the group consisting of:

a) the DNA sequence of Figure 1;

b) DNA sequences which hybridize to the DNA sequence of Figure 1 and which code for a polypeptide exhibiting mammalian multi-lineage cellular growth factor activity and/or mammalian mast cell growth factor activity; and

c) DNA sequences which on expression code for a protein exhibiting mammalian multi-lineage cellular growth factor activity and/or mammalian mast cell growth factor activity.

14. A polypeptide exhibiting human multi-cellular growth factor activity and/or human mast cell growth factor activity, whose DNA coding sequence is capable of hybridizing with DNA coding for murine IL-3, especially such a polypeptide that is capable of acting on a hematopoietic cell line, in particular one produced by cultivating the organism or cell of claim 12.

15. Mammalian IL-3, e.g. murine IL-3, substantially free of other mammalian proteins.

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                                              1           20            40              60
GGGGGGGGGG GGGAACCCCT TGGAGGACCA GAACGAGACA ATG GTT CTT GCC AGC TCT ATC CAC ACC ATG CTG CTC
                                             MET Val Leu Ala Ser Ser Ile His Thr MET Leu Leu


          80           100           120           140
CTG CTC CTG ATG CTC TTC CAC CTG GGA CTC CAA GCT CTC AGT ATC GGC GAT ACC CAC CGT TTA ACC AGA ACG TTG
Leu Leu Leu MET Leu Phe His Leu Gly Leu Gln Ala Leu Ser Ile Gly Asp Thr His Arg Leu Thr Arg Thr Leu


          160           180           200           220
AAT TGC AGC TCT ATT GTC AAG GAG ATT ATA GGG AAG CTC CCA GAA CCT GAA CTC AAA ACT GAT GAA GGA CCC TCT
Asn Cys Ser Ser Ile Val Lys Glu Ile Ile Gly Lys Leu Pro Glu Pro Glu Leu Lys Thr Asp Glu Gly Pro Ser


          240           260           280           300
CTG AGG AAT AAG AGC TTT CGG AGA GTA AAC CTG TCC AAA TTC GTG GAA AGC GTA GAA CAA GGA GAA GTG GAT CCT GAG GAC AGA
Leu Arg Asn Lys Ser Phe Arg Arg Val Asn Leu Ser Lys Phe Val Glu Ser Val Glu Gln Gly Glu Val Asp Pro Glu Asp Arg


          320           340           360           380
TAC GTT ATC AAG TCC CCT ACA TCT GCG AAT GAC TCT GGG CTG CCA ACA TCT TGT TGC CTG GCG CTG CCA GGG GTC
Tyr Val Ile Lys Ser Pro Thr Ser Ala Asn Asp Ser Gly Leu Pro Thr Ser Cys Cys Leu Ala Leu Pro Gly Val


          400           420           440           460
TTC ATT CGA GAT CTG GAT TTT CGG AAG AAA CTG AGA TTC TAC ATG GTC CAC CTT AAC GAT CTG GAG ACA GTG CTA
Phe Ile Arg Asp Leu Asp Phe Arg Lys Lys Leu Arg Phe Tyr Met Val His Leu Asn Asp Leu Glu Thr Val Leu


          480           500           520
GCC TCT AGA CCA CCT CAG CCC GCA TCT GGC TCC GTC TCT CCT AAC CGT GGA ACC GTG GAA TGT TAA .
Ala Ser Arg Pro Pro Gln Pro Ala Ser Gly Ser Val Ser Pro Asn Arg Gly Thr Val Glu Cys
```
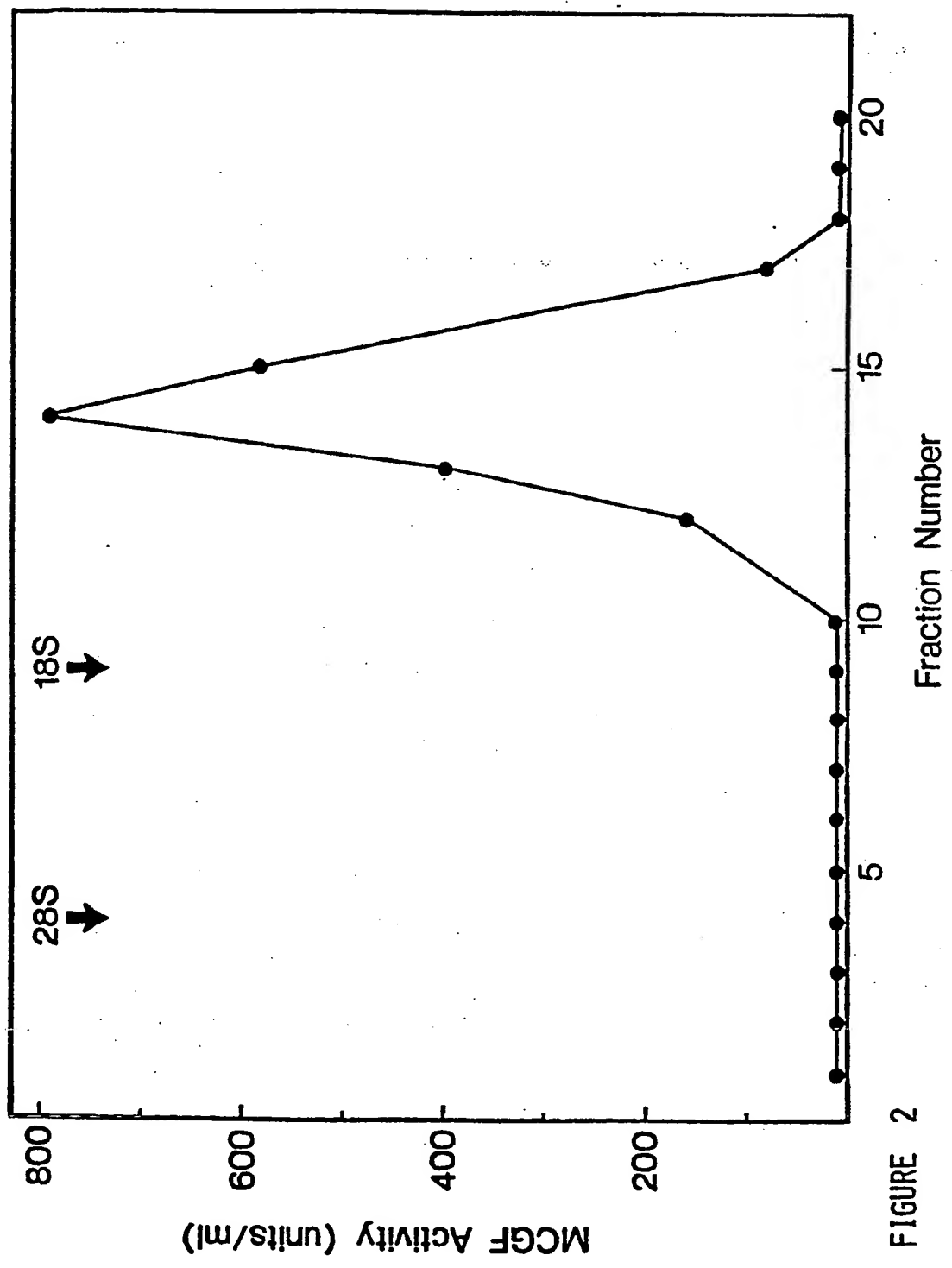
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4
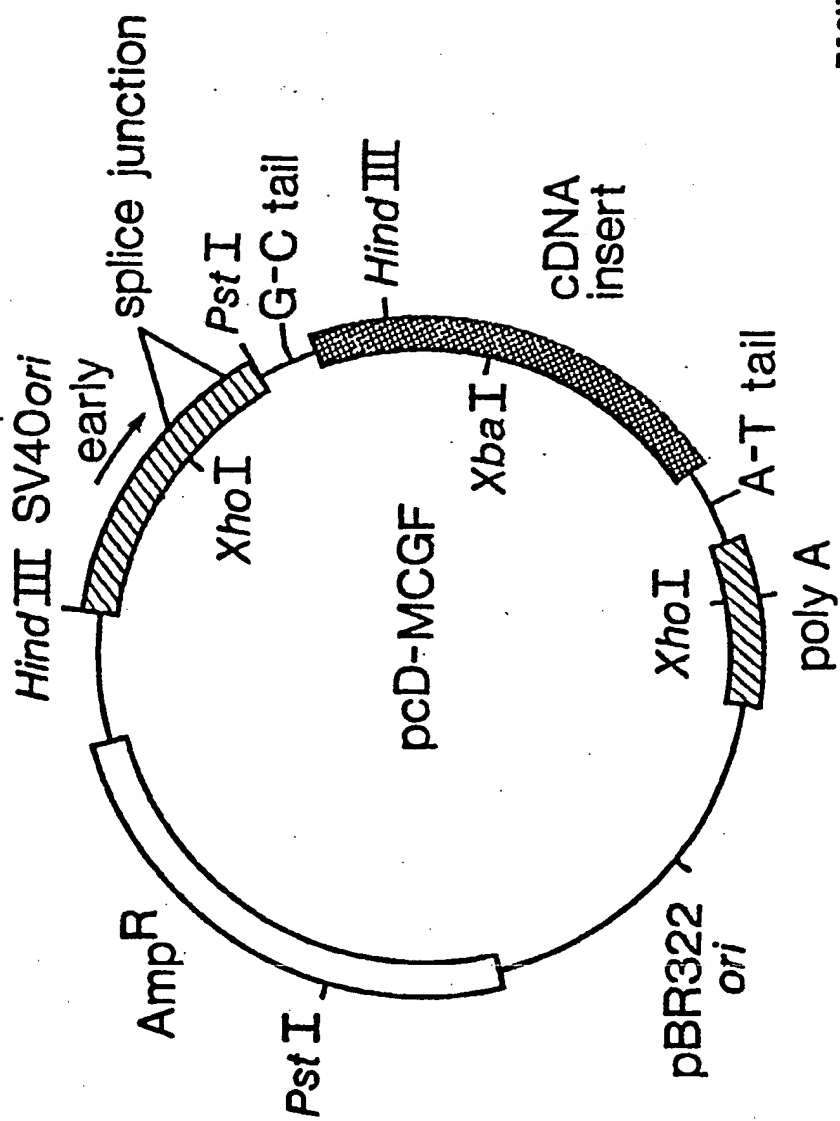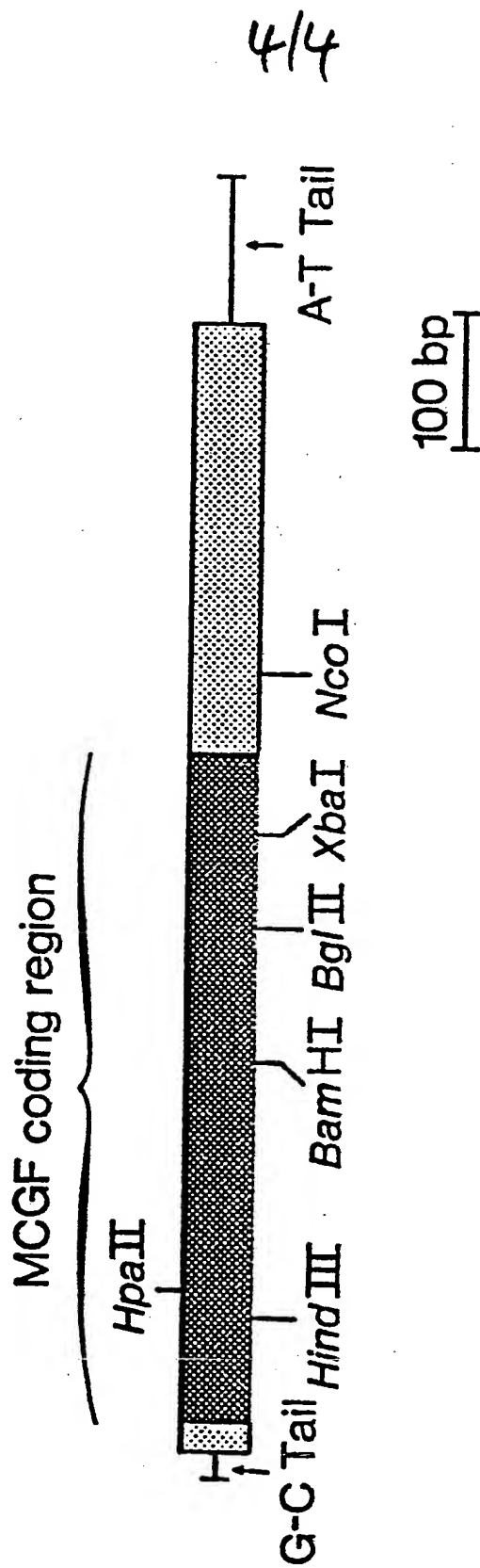
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
	DOCUMENTS CONSIDERED TO BE RELEVANT		EP 84111677.5
A,P	EP - A1 - 0 091 539 (AJINOMOTO). * Claims 1,2,19,20,23-25,28-30 *	1-4,7, 10-13	C 12 N 15/00 C 07 K 15/06 C 07 H 21/04
A,D	JOURNAL OF CELLULAR PHYSIOLOGY SUPPLEMENT, vol. 1, 1982, Philadelphia N.N. ISCOVE et al. "Molecules Stimulating Early Red Cell, Granulocyte, Macrophage, and Megakaryocyte Precursors in Culture: Similarily in Size, Hydrophobicity, and Charge" pages 65-78 * Page 65 - page 66, column 1, line 35. *	5,14, 15	C 12 P 21/00 A 61 K 37/02
A,D	THE JOURNAL OF IMMUNOLOGY, vol. 131 no. 1, July 1983, Baltimore USA J.N. IHLE "Biologic Properties of Homogeneous Interleukin 3. 1. Demonstration of WEHI-3 Growth Factor Activity, Mast Cell Growth Factor Activity, PCell-Stimulating Factor Activity, Colony-Stimulating Factor Activity, and Histamine-Producing Cell-Stimulating Factor Activity" pages 282-287 * Pages 285-287 (discussion) *	5,14, 15	**TECHNICAL FIELDS SEARCHED (Int. Cl.4)** C 12 N C 07 K C 07 H C 12 P A 61 K
A	EP - A2 - 0 077 571 (AJINOMOTO)		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	10-01-1985	FARNIOK